Allele Mining and Sequence Diversity at the Wheat Powdery Mildew Resistance Locus *Pm3*

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I. Summary

Wheat is one of the most important food crops for humans. Wheat diseases cause severe yield losses and often reduce grain quality. Powdery mildew of wheat is one of the major foliar diseases of wheat caused by the fungus *Blumeria graminis f.sp. tritici*. Disease resistance genes offer effective disease control and have been used extensively in breeding. Until now, 36 *Pm* genes (*Pm1* to *Pm36*) have been genetically mapped on different chromosomal regions, but *Pm3* is the only wheat powdery mildew resistance gene cloned. Recently, the seven different known *Pm3* resistance alleles (*Pm3a* to *Pm3g*) conferring race specific resistance to different powdery mildew isolates were isolated. These alleles have all been used in breeding. As resistance may be lost with rapid emergence of new pathogen strains, it becomes a continuous task to identify new resistance genes and to transfer these genes into common wheat if they are present in wild relatives. Non- explored genetic resources secured in gene banks potentially have additional alleles for resistance and tolerance to diseases, pests and harsh environments. This diversity remains largely unexplored at the molecular level due to a lack of fast and efficient tools to identify and study potential new alleles. Therefore, it is essential to develop strategies to assemble focused sets of germplasm material for molecular diversity of specific traits. A new approach called Focused Identification of Strategy (FIGS) was recently suggested which can improve the efficiency of accessing the genetic resources.

The aim of this thesis was to efficiently screen the wheat genetic resources using FIGS subsets for allele mining at the wheat powdery mildew resistance locus *Pm3*. This study also included analysis of sequence diversity available at the *Pm3* locus among the hexaploid and tetraploid wheat species. In the first part of work, we carried out a large-scale systematic allele-mining from hexaploid wheat landraces using molecular tools derived from the *Pm3* gene sequence. We characterized the “FIGS powdery mildew set” by using a combined

strategy of screening for genetic diversity with *Pm3* specific molecular markers and classical pathogenicity tests. We screened the identified powdery mildew resistant lines for the occurrence of already known *Pm3* alleles by using allele specific markers. *Pm3b* was found to be the most frequent allele, followed by *Pm3c* while the other alleles were not found in the subset. This analysis led to the identification of powdery mildew resistant lines which possess a *Pm3* like gene but not a known allele of *Pm3*. From 45 of these resistant landraces, we could isolate 16 new *Pm3* allelic sequences. The differences within the new alleles and in comparison to known *Pm3* alleles were mainly found in the LRR domain. Functional analysis using both virus-induced gene silencing and transient transformation assays, was used to identify seven functionally active, new *Pm3* alleles out of the 16 candidates. These *Pm3* alleles, *Pm3l, Pm3m, Pm3n, Pm3o, Pm3p, Pm3q* and *Pm3r* now extend the previously known *Pm3* allelic series. Two of these, *Pm3_42416/Pm3l* and *Pm3_42255/Pm3m* were further studied in detail for resistance activity and specificity. We conclude that at least these two alleles isolated from landraces are slow acting alleles while the known *Pm3* alleles are known to provide a rapid resistance response. These results suggest that slow resistance is a general phenomenon of *Pm3* genes in landraces and the reason why these genes were never selected for breeding.

In the second part of the work, we have screened a collection of wild and domesticated tetraploid wheat lines for *Pm3* genes and studied their genetic and geographical differentiation. The identification of 24 *Pm3* haplotypes from tetraploid wheat and the conservation of the *Pm3* locus at two different ploidy levels in the wheat gene pool allowed to correlate and to link the evolution of this gene in relation to the history of wheat evolution and domestication. Among the 3 screened tetraploid gene pools (wild *T. dicoccoides*, domesticated *T. dicoccum* and *T. durum*) , most *Pm3* alleles were detected in wild *T.*

dicoccoides accessions indicating that the wild gene pool is enriched in *Pm3* sequences compared to the domesticated emmer. Phylogenetic analysis of *Pm3* haplotypes from tetraploid wheat reflected a relatively more ancient divergence compared to bread wheat alleles and is compatible with the estimated time of the first hybridization event at the origin of tetraploid wheat, around 0.5 MYA. Functional *Pm3* resistance alleles were not very frequently found in the tetraploid gene pool although it was possible to identify one functional *Pm3* allele, now called *Pm3k*. We propose that low frequency of *Pm3*-based resistance in wild emmer is due to a recent and independent evolution of functional *Pm3* resistance genes in the tetraploid vs. hexaploid wheat species.

III. Zusammenfassung

Weizen ist eines der wichtigsten Getreide für die menschliche Ernährung. Weizenkrankheiten verursachen schwerwiegende Ertragsverluste und verringern oft die Kornqualität. Eine der bedeutsamsten Blatterkrankungen des Weizens ist der durch den Pilz *Blumeria graminis f.sp. tritici* verursachte Weizenmehltau. Krankheitsresistenzgene erlauben eine effiziente Kontrolle der Krankheit und wurden dementsprechend intensiv in der Züchtung benutzt. Bis heute wurden 36 *Pm* Weizenmehltau-Resistenzgene (*Pm1* bis *Pm36*) in verschiedenen Chromosomenregionen genetisch kartiert, wovon bisher jedoch einzig *Pm3* kloniert wurde. Kürzlich wurden die sieben bekannten *Pm3* Resistenzallele (*Pm3a* bis *Pm3g*), welche rassenspezifische Resistenz gegenüber unterschiedlichen Mehltauisolaten verleihen, isoliert. Alle diese Allele wurden in der Züchtung verwendet. Weil die Resistenz mit dem raschen Auftreten von neuen Pathogenstämmen verloren gehen kann, müssen fortlaufend neue Resistenzgene identifiziert werden. Wenn diese neuen Resistenzgene in verwandten Wildarten vorkommen, müssen sie zudem in Brotweizen transferiert werden. Noch nicht untersuchte genetische Ressourcen in genbanken tragen möglicherweise weitere zusätzliche Allele für die Resistenz und Toleranz gegenüber Pflanzenschädlingen und widrigen Umwelteinflüssen. Auf der molekularen Ebene ist diese Diversität bisher jedoch grösstenteils unerforscht, weil rasche und effiziente Ansätze zur Identifikation und Untersuchung neuer Allele fehlen. Es ist deshalb wichtig, Strategien zu entwickeln, um eine fokussierte Sammlung genetischer Diversität für spezifische Eigenschaften zusammenzustellen. Kürzlich wurde mit „Fosused Identification of Strategy“ (FIGS) ein neuer Ansatz vorgeschlagen, welcher den effizienten Zugriff auf genetische Ressourcen verbessern kann.

Das Ziel der vorliegenden Dissertation war ein effizientes Durchsuchen der genetischen Weizenressourcen mittels FIGS Untermengen, um neue Allele am Weizenmehltau-Lokus *Pm3* zu identifizieren. Zudem wurde die Sequenzdiversität am *Pm3* Lokus in hexa- und

tetraploiden Weizenarten untersucht. Im ersten Teil der Arbeit wurde mithilfe der molekularen Information der *Pm3* Gensequenz eine umfangreiche systematische Suche nach Allelen in hexaploiden Weizenlandrassen durchgeführt. Durch eine kombinierte Strategie von systematischer Suche nach genetischer Diversität mit *Pm3*-spezifischen molekularen Markern und klassischen Pathogenizitätstests wurde das „FIGS Mehltau-Set“ charakterisiert. Die identifizierten Mehltau-resistenten Linien wurden durch Allel-spezifische Marker auf bereits bekannte *Pm3* Allele hin untersucht. Das häufigste gefundene Allel war *Pm3b* gefolgt von *Pm3c*. Die anderen Allele wurden in der Untermenge nicht gefunden. Diese Analyse führt zur Identifizierung von Mehltau-resistenten Linien, welche *Pm3*-ähnliche Gene, aber kein bekanntes *Pm3* Allel besitzen. Von 45 dieser resistenten Landrassen konnten 16 neue *Pm3*-allelische Sequenzen isoliert werden. Unterschiede zwischen den neuen Allelen und im Vergleich zu den bekannten *Pm3* Allelen fanden sich hauptsächlich in der LRR Domäne. Durch die funktionale Analyse mittels Virus-induziertem Ausschalten und transienter Transformation wurden unter den 16 Kandidaten sieben funktional aktive, neue *Pm3* Allele identifiziert. Diese *Pm3* Allele, *Pm3l, Pm3m, Pm3n, Pm3o, Pm3p, Pm3q und Pm3r,* erweitern nun die bisher bekannte *Pm3* Allelserie. Zwei der Allele, *Pm3_42416/Pm3l* und *Pm3_42255/Pm3m,* wurden detaillierter auf Resistenzaktivität und –spezifität hin untersucht. Wir ziehen die Schlussfolgerung, dass wenigstens diese zwei aus Landrassen isolierten Allele langsam reagierende Allele sind, während man von den bekannten *Pm3* Allele weiss, dass sie eine rasche Resistenzreaktion hervorzurufen. Diese Resultate deuten darauf hin, dass langsame Resistenz ein generelles Phänomen der *Pm3* Gene in Landrassen ist, und diese Gene deshalb nie für die Züchtung ausgewählt wurden.

Im zweiten Teil der Studie wurde eine Sammlung von wilden und domestizierten tetraploiden Weizenlinien nach *Pm3* Genen durchsucht und deren genetische und geographische Differenzierung untersucht. Die Identifizierung von 24 *Pm3* Haplotypen in tetraploidem

Weizen und die Konservierung des *Pm3* Lokus auf zwei verschiedenen Ploidiestufen im Weizengenpool erlaubten es, die Evolution dieses Genes mit der Geschichte der Weizenevolution und –domestizierung in Korrelation zu setzen. In den drei untersuchten tetraploiden Genpools (wilder *T. dicoccoides*, domestizierter *T. dicoccum* und *T. durum*) wurden die meisten *Pm3* Allele in wilden *T. dicoccoides* Linien gefunden. Dies deutet darauf hin, dass der wilde Genpool im Vergleich zum domestizierten Emmer mehr *Pm3* Sequenzen enthält. Die phylogenetische Untersuchung von *Pm3* Haplotypen aus tetraploidem Weizen widerspiegelte eine relativ ältere Divergenz im Vergleich zu Allelen des Brotweizens und ist kompatibel mit dem Zeitpunkt der ersten Hybridisierung am Ursprung von tetraploidem Weizen vor rund 0.5 Millionen Jahren. Funktionale *Pm3* Resistenzallele sind nicht sehr häufig im tetraploiden Genpool, obwohl es möglich war, ein funktionales *Pm3* Allel, welches nun *Pm3k* genannt wird, zu identifizieren. Es wird vermutet, dass das seltene Vorkommen von *Pm3*-basierter Resistenz in wildem Emmer in einer kürzlichen und unabhängigen Evolution von funktionalen *Pm3* Resistenzgenen in tetraploiden gegenüber hexaploiden Weizenarten gründet.

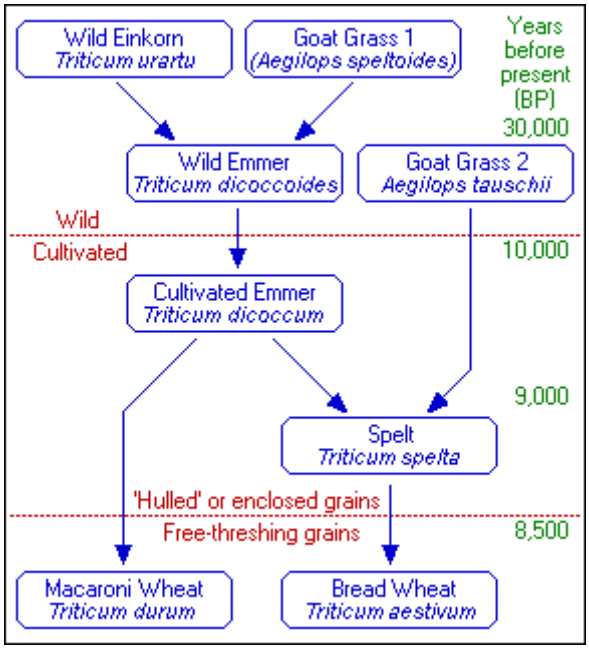
III. General Introduction

1. Wheat as a crop and its origin

Wheat is one of the three most important global food crops, the other two being maize and rice. The world's main wheat producing regions span from the central plains of USA and Canada, Europe, Russia, India, China, to Australia and back to the southern parts of the American continent. It is major food crop because of the plant's agronomic adaptability, ease of grain storage and ease of converting grain into flour for making breads, cookies, cakes, pasta etc. Wheat is an important source of carbohydrate in a majority of countries and also contains minerals, vitamins and fats (lipids) that are important for human nutrition.

Wheat (*Triticum aestivum*) is an allopolyploid species containing three distinct, homoeologous genomes A, B and D. It evolved through two independent and spontaneous hybridization events which brought together the sub-genomes A, B and D. The diploid einkorn wheat (*T. monococcum*) was probably the first wheat species to be widely cultivated ~10,000 years ago in south eastern Turkey. Currently, it has very little agricultural significance but it is still cultivated as animal feed in some regions of Turkey, Italy and Spain (Feuillet *et al*, 2007). The first hybridization event occurred when a still unknown species (BB) closely related to *Aegilops speltoides* (SS) hybridized with wild diploid *Triticum urartu* (AA). This led to formation of tetraploid wheat (*Triticum turgidum*; AABB). One sub species, *Triticum turgidum* ssp. *durum*, gave rise to pasta cultivars of today (Figure 3.1). A second evolutionary event was the hybridization of *T. turgidum* with the wild diploid species *Aegilops tauschii* (DD), which led to the formation of hexaploid bread wheat (*Triticum aestivum*; AABBDD). Hexaploid bread wheats account for ~90% of world wheat production (Feuillet *et al*, 2007).

Figure 3.1: Evolution of hexaploid bread wheat



Source: http://www.newhallmill.org.uk/wht-evol.htm

2. Wheat diseases

Wheat production is threatened by a constantly changing population of pathogen species and races. Wheat diseases cause severe yield losses, often reduce grain quality and are mostly caused by fungal pathogens. Some of the most important fungal diseases of wheat include the three rust species (stripe, leaf and stem rust), powdery mildew, fusarium, septoria, mycosphaerella and tan spot. Powdery mildew is one of the most destructive foliar diseases and is caused by the fungus *Blumeria graminis f.sp. tritici*, which is an obligate biotrophic parasite.

2.1 Powdery mildew of wheat: symptoms, severity and control

The quite distinctive symptoms of the disease include whitish cottony growth of conidia and mycelia mainly on the leaf surfaces but can also be found on stems and heads. The fungus

grows primarily on the surface of the host mainly in epidermal cells. Within a few days of infection, white powdery pustules become visible. These pustules produce long chains of small, asexually produced conidia, which are easily dispersed by wind. The optimum conditions for the growth of powdery mildew are temperatures between 15°C and 25°C and a relative humidity ranging from 80 to 100%. Optimally, new pustules with conidia are produced every seven to ten days. During aging, the mycelium becomes denser and turns grayish. Towards maturity of the crop, mildew forms the fruiting bodies called cleistothecia. Cleistothecia can remain attached to the straw of harvested crop and this stage is referred to as the sexual stage of the fungus. Cleistothecia contain survival structures called ascospores which later serve to infect fresh wheat leaves. The newly planted wheat is infected with the spores developing within cleistothecia (Figure 3.2).

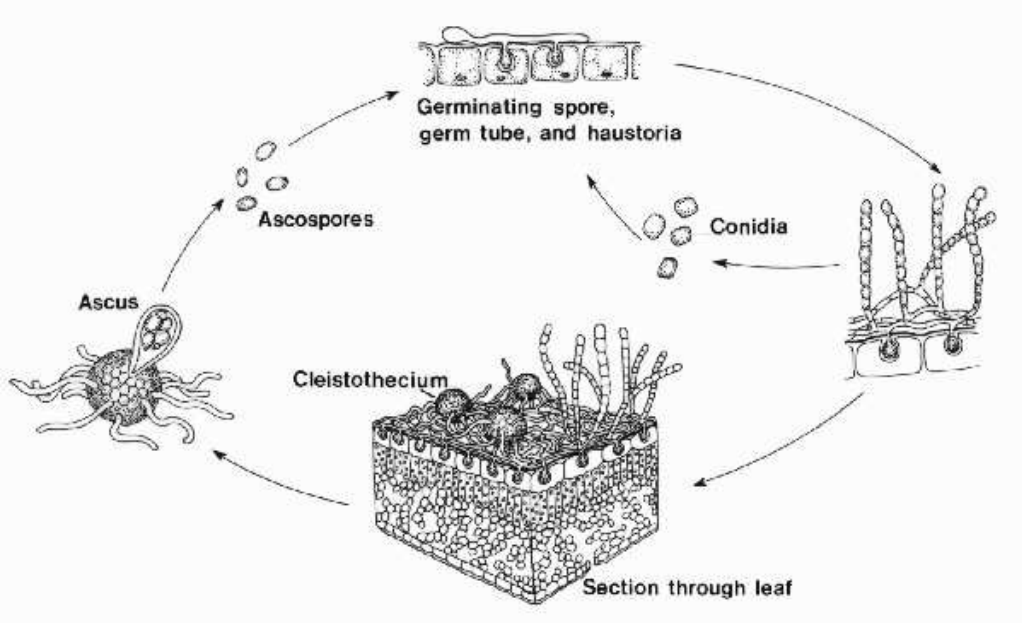


Figure 3.2: Schematic representation of the disease cycle for the powdery mildew fungus *Blumeria graminis*.
Source: The American Phytopathological Society.
(http://www.apsnet.org/education/k-12plantpathways/TeachersGuide/Activities/PowderyMildew/DIAGRAMS3.HTM)

The powdery mildew attack is known to result in reduced kernel size and weight, and ultimately lower yield. Disease severity can vary depending on weather conditions, susceptibility of the cultivars as well as the densities of the leaf canopies (Lipps and Madden, 1989). Disease severity has been reported to increase with the increased levels of nitrogen fertilizer applications which provide dense stands and green tissue, both favourable for pathogen growth. Yield losses occurring due to powdery mildew may be as high as 45% (Hsam and Zeller, 2002).

Wheat powdery mildew occurs in the form of distinct races and new forms continue to be formed as a result of genetic recombination. The fungus is highly host-specific i.e., the isolates which attack wheat do not attack other crops such as barley (http://wheatdoctor.cimmyt.org). Powdery mildew occurs in cool, humid, and semiarid environments almost wherever wheat is grown (Cunfer, 2002). It has been reported worldwide for example in Europe, Africa, America, Asia, and Australia. The suggested methods to control powdery mildew include the implementation of practices like scarce planting, crop rotation, application of fungicides and use of resistant cultivars. However, use of fungicides is not considered to be very environmental friendly and is not an economical option. In this situation, resistant wheat varieties remain the most effective solution for disease control.

3. Disease resistance genes

Plant disease resistance genes (*R* genes) are a major component of the plant response to pathogen attacks. Disease resistance is frequently mediated through recognition between pathogen avirulence genes and plant resistance genes. The common defense responses in

plants also include callose and lignin deposition, salicylic acid synthesis, and hypersensitive response leading to cell death (Hammond-Kosack and Jones, 1997), thus, restricting the pathogen growth. Evolution of disease resistance genes is mediated through mechanisms such as recombination between alleles/genes, point mutations, gene conversion events and illegitimate recombination (Michelmore and Meyers, 1998; Kuang *et al*, 2004, Wicker *et al*, 2007).

In case of powdery mildew, mostly single resistance genes confer complete resistance but are only active against specific races of the pathogen. This race-specific resistance is based on the recognition event between the product of the plant disease resistance gene and the corresponding avirulence gene of the pathogen (Flor, 1971).

3.1 Race specific resistance in host pathogen interactions

H. H. Flor (1971) introduced the classical gene-for-gene model for disease resistance in plants on the basis of his pioneering genetic studies of the interaction between flax and flax-rust. According to this model, a specific resistance (*R*) gene product interacts with a specific avirulence (Avr) gene product from the pathogen, triggering a plant resistance reaction. The resistance reaction is often caused by a genetically programmed death of infected cells (the hypersensitive response, HR), as well as local accumulation of antimicrobial compounds at the site of infection. The gene-for-gene hypothesis was later modified to a receptor-ligand model in which the *R* protein functions as receptor and the corresponding Avr protein as ligand wherein binding of the ligand to the receptor triggers defense response. However, the lack of evidence for a direct interaction between most *R* proteins and their elicitors, together with the identification of plant proteins that directly interact with both Avr and *R* proteins, led to a new interpretation of the gene-for-gene model: the guard hypothesis (Van der Biezen and

Jones, 1998; Van der Hoorn *et al*, 2002). This model suggests that *R* proteins guard the proteins (called "guardees") which are targets of Avr proteins. The interaction between the Avr factor and the guardee is sensed by the *R* proteins that subsequently triggers defense responses. The model implies that *R* proteins function in recognition complexes containing their guardees.

Thus, resistance proteins directly or indirectly interact with pathogen emitted effector molecules and trigger a resistance reaction (DeYoung and Innes, 2006). It has been hypothesized that plant trans-membrane proteins are capable of recognizing pathogen associated molecular patterns (PAMPs) such as bacterial flagellin, lipopolysaccharides etc. This activates basal defense responses which are now referred to as PTI (PAMP triggered immunity; Jones and Dangl, 2006). It has been postulated that a biological arms race is occurring between plants and pathogens. According to this, pathogen populations are subjected to selection pressure and evolve effector molecules in order to evade basal plant defense. Subsequently, plants evolve resistance proteins to detect specific effector molecules to provide a second line of defense referred to as effector-triggered immunity (ETI; Jones and Dangl, 2006; De Young and Innes, 2006). Most of the disease resistance genes encode proteins with a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) domain associated at the N-terminus either with a Toll-interleukin receptor-like (TIR) domain or a coiled-coil (CC) domain (Jones and Dangl, 2006). The NBS domain of plant resistance proteins is suggested to be responsible for binding and hydrolysis of ATP and GTP (Tameling *et al*, 2002) whereas the LRR region was shown to play a major role in pathogen recognition specificity (Ellis *et al*, 1999). The wheat powdery mildew resistance gene *Pm3* also encodes CC-NBS-LRR type of resistance proteins (Yahiaoui *et al*, 2004).

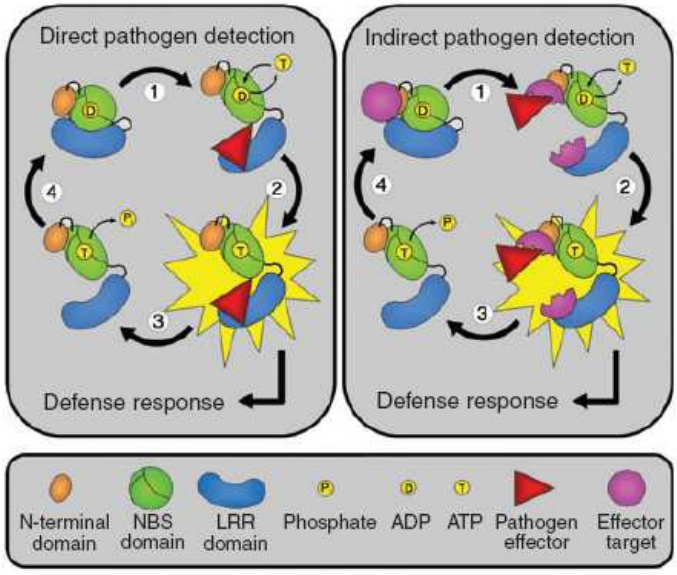


Figure 3.3. Model for plant NBS-LRR activation (De Young and Innes, 2006).

Signaling is activated in a similar way for both direct (left) and indirect (right) modes of pathogen detection. Presence of the pathogen effector (1) alters the structure of the NBS-LRR protein through direct binding (left) or modification of additional plant proteins (right), allowing exchange of ADP for ATP. Binding of ATP to the NBS domain (2) results in activation of signal transduction through the creation of binding sites for downstream signaling molecules and/or the formation of NBS-LRR protein multimers. Dissociation of the pathogen effector and modified effector targets (if present; 3) along with hydrolysis of ATP (4) returns the NBS-LRR protein to its inactive state.

De Young and Innes (2006) suggested a model for the role of NBS-LRR proteins in direct and indirect pathogen recognition (Figure 3.3). For indirect pathogen recognition, proteins targeted by pathogen effectors exist in a complex together with the amino-terminal domain, the NBS and LRR domains. The effector induces a conformational change in NBS-LRR protein, thus enabling the change of ADP for ATP. These changes probably result in creation of new binding sites for downstream signaling molecules, resulting in the activation of

signaling pathways. Whereas the pathogen effector molecules directly bind to NBS-LRR protein in the direct interaction model, the LRR domain may be involved in effector binding. After effector binding, the signal transduction is probably activated through conformational changes, as is the case for indirect recognition.

3.2 Sources of powdery mildew resistance genes

The introduction of resistance genes from wild relatives, e.g. the diploid and tetraploid progenitors of hexaploid wheat (Table 3.1), is known to be successful in broadening wheat disease resistance *(*Miranda *et al,* 2006; Liu *et al*, 2002; Rong *et al,* 2000). Some examples include *Aegilops speltoides (Pm32), T. timopheevi* (*Pm6*), *Triticum turgidum var dicoccoides* (*Pm26, Pm30*) and *Pm34* from *Aegilops tauschii Coss.* (Allard & Shands, 1954; Miranda *et al,* 2006; Liu *et al,* 2002; Rong *et al,* 2000). *Pm* genes are also known to have been identified from widely distributed traditional wheat cultivars and landraces. Over thousands of years, landraces of hexaploid wheat have developed under a variety of different edaphic and climatic environments. This has resulted in the evolution of a large number of ecotypes adapted to specific local environments.

Common wheat has also been genetically improved through the introgression of rye chromatin. The rye chromosome arm 1RS is the most widely incorporated alien variation in the wheat genome. Wheat cultivar “Amigo” carries the powdery mildew resistance gene *Pm17* on its introgressed 1RS chromosome arm (Forsstrom and Merker, 2001). Traditionally, resistance genes in wild relatives of wheat have been introgressed by complex breeding schemes involving irradiation and chromosomal translocations (Baum *et al*, 1992) which also led to linkage drag.

Different molecular marker techniques, cytogenetic analysis and analysis of host- pathogen interactions have been used to map and determine chromosomal locations of *Pm* genes in wheat. Monosomic analysis has been the most commonly used method to assign genes to specific chromosomes (Zeller *et al*, 1993). To date, 36 *Pm* genes have been assigned (Table 3.1) to different chromosomes. There are *Pm* genes known to possess more than one resistant allele for example, *Pm1* with 4 alleles (Hsam *et al*, 1998), *Pm3* with 7 alleles (Briggle, 1966; Zeller *et al*, 1993; Yahiaoui *et al*, 2006), *Pm4* with 2 alleles (The *et al*, 1979) and *Pm5* with 4 alleles (Hsam *et al*, 2001). Hsam and Zeller (2002) stated that the loci *Pm10*, *Pm11*, *Pm14* and *Pm15* contain individual genes for resistance against *Erysiphe graminis* f.sp. *agropyri* and are not effective against *Blumeria graminis* f.sp. *tritici*.

Despite these efforts in identifying diverse resistance in the germplasm, the genetic base of disease resistance in wheat remains dangerously narrow and adaptation of pathogens is always a threat, challenging the resistance of existing elite material. Currently, the emergence and spread of the new virulent stem rust race *Ug99* is considered to be a potential threat to wheat production worldwide. The *Ug99* race has overcome the major stem rust resistance gene *Sr31* (http://www.ars.usda.gov/Main/docs.htm?docid=14649). Previously, there was no report on virulence against *Sr31*, a gene which is widely used in India, China, Europe and South America.

Thus, it is essential to identify new sources of resistance that might exist in germplasm collections and then to make combinations with existing sources to develop durable resistance. The molecular isolation of the underlying genes or alleles of respective genes and their use through transgenic or back crossing technologies will contribute to an efficient future use of resistance.

Table 3.1 : Powdery mildew resistance genes: Source and chromosomal locations

Gene/ allele	Location	Source	Cultivar/line	Reference
Pm1a	7AL	*Triticum aestivum*	Axminster	Sears and Briggle (1969)
Pm1b	7AL	*T. monococcum*	MocZlatka	Hsam et al. (1998)
Pm1c	7AL	*T. monococcum*	Weihenst. MIN	Hsam et al. (1998)
Pm1d	7AL	*T. spelta*	*T. spelta* var *duhamelianum*	Hsam et al. (1998)
Pm2	5D	*Aegilops tauschii*	XX194	Lutz et al. (1995)
Pm3a	1AS	*T. aestivum*	Asosan	Briggle and Sears (1966)
Pm3b	1AS	*T. aestivum*	Chul	Briggle (1966)
Pm3c	1AS	*T. aestivum*	Sonora	Briggle (1966)
Pm3d	1AS	*T. aestivum*	Kolibri	Zeller et al. (1993)
Pm3e	1AS	*T. aestivum*	W150	Zeller et al. (1993)
Pm3f	1AS	*T. aestivum*	Michigan amber	Zeller et al. (1993)
Pm3g	1AS	*T. aestivum*	Aristide	Zeller and Hsam (1998)
Pm4a	2AL	*T. dicoccum*	Khapli/8*Chancellor	The et al. (1979)
Pm4b	2AL	*T. carthlicum*	Armada	The et al. (1979)
Pm5a	7BL	*T. dicoccum*	Hope	Law and Wolfe (1966)
Pm5b	7BL	*T. aestivum*	Ibis	Hsam et al. (2001)
Pm5c	7BL	*T. aestivum* ssp. *sphaerococcum*	Kolandi	Hsam et al. (2001)
Pm5d	7BL	*T. aestivum*	IGV 1-455	Hsam et al. (2001)
Pm6	2B	*T. timopheevii*	TP111	Jorgensen and Jensen (1973)
Pm7	4BS.4BL-2RL	*Secale cereale*	Transec	Friebe et al. (1996)
Pm8	1BL.1RS	*S. cereale*	Disponent	Hsam and Zeller (1997)
Pm9	7A	*T. aestivum*	N14	Hsam et al. (1998)
Pm12	6BS-6SS.6SL	*A. speltoides*	Trans.line31	Miller et al. (1988)
Pm13	3BL.3BS-3S	*A. longissima*	C.S.trans.line	Biagetti et al. (1998)
Pm16	4A	*T. dicoccoides*	Norman rec.line	Reader and Miller (1991)
Pm17	1AL.1RS	*S. cereale*	Amigo	Heun et al. (1990)
Pm19	7D	*A. tauschii*	XX186	Lutz et al. (1995)
Pm20	6BS.6RL	*S. cereale*	MIP6L	Friebe et al. (1994)
Pm21	6VS.6AL	*Haynaldia villosa*	Yangmai 5 line	Chen et al. (1995)
Pm22	1D	*T. aestivum*	Virest	Peusha et al. (1996)
Pm24	1D	*T. aestivum*	Chiyacao	Huang et al. (2000)
Pm25	1A	*T. monococcum*	NC96BGTA5	Shi et al. (1998)
Pm26	2BS	*T. dicoccoides*	TTD140	Rong et al. (2000)
Pm27	6B-6G	*T. timopheevii*	146-155	Järve et al. (2000)
Pm28	1B	*T. aestivum*	Meri	Peusha et al. (2000)
Pm29	7DL	*A. ovata*	Pova	Zeller et al. (2002)
Pm30	5BS	*T. dicoccoides*	C20	Liu et al. (2002)
Pm31	6A	*T. dicoccoides*	G-305-M	Xie et al. (2004)
Pm32	T1BL.1SS	*A. speltoides*	L501	Hsam et al. (2003)
Pm34	5D	*A. tauschii*	NC97BGTD7	Miranda et al. (2006)
Pm35	5DL	*A. tauschii*	NC96BGTD3	Miranda et al. (2007)
Pm36	5B	*T. dicoccoides*	MG29896	Blanco et al. (2008)

Modified from Hsam and Zeller (2002)

3.3 Molecular characterization and cloning of disease resistance genes in wheat

The wheat genome is large (16 x 10^9 bp) and the major fraction consists of repetitive sequences, thus, the molecular cloning of genes, for which only genetic information is available, remains a challenge (Keller *et al*, 2005). Therefore, the first efforts to characterize loci of disease resistance at the molecular level concentrated on the development of molecular markers linked to these important traits. The availability of molecular markers linked to specific resistance genes and of information on their genetic location has supported resistance breeding by simplifying the detection of specific genes in breeding material. This makes the selection process faster and more cost effective. The range of molecular markers used for this purpose includes restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), sequence tagged sites (STS), simple sequence repeats (SSR)/ microsatellites, expressed sequence tags (ESTs) and RGAs (resistance gene analogs). SSR/microsatellite markers are highly polymorphic in wheat and are now widely used in wheat genetics for diversity studies, genotype identification (Prasad *et al*, 2000), marker-assisted selection, mapping, identification and tagging of disease resistance genes (Chagué *et al*, 1999; Miranda *et al*, 2006; Liu *et al*, 2002; Adhikari *et al*, 2003). Recently, microsatellite markers have been found linked to leaf rust resistance gene *Lr34* (Bossolini *et al*, 2006) supporting the identification of wheat genotypes with *Lr34*.

To design precise and targeted molecular tools for diversity analysis, knowledge on the DNA sequence at a particular resistance locus becomes important, requiring the molecular cloning of genes. In map-based cloning, saturation of the genomic region of interest is greatly supported by genomic information and markers obtained from the grass model species rice

and brachypodium (Griffiths *et al*, 2006). In addition, there are now a number of BAC libraries available from diploid, tetraploid and hexaploid wheat species (Keller *et al*, 2005) supporting map-based cloning strategies of wheat genes. DNA sequence information obtained from cloning is essential to devise rapid and inexpensive PCR strategies to isolate alleles of identified resistance genes from a wide range of cultivars, landraces and related species.

Until now, three leaf rust resistance genes (*Lr*) and one allelic series of a powdery mildew (*Pm*) resistance gene have been cloned from wheat: *Lr21* (Huang *et al*, 2003), *Lr10* (Feuillet *et al*, 2003), *Lr1* (Cloutier *et al*, 2007) and the *Pm3* alleles (Yahiaoui *et al*, 2004; Srichumpa *et al*, 2005; Yahiaoui *et al*, 2006). Out of the 36 *Pm* genes characterized, *Pm3* is the only wheat powdery mildew resistance gene cloned till date (Yahiaoui *et al*, 2004). The *Pm3* gene exists in seven different alleles (*Pm3a-Pm3g*) and these alleles confer resistance to specific races or isolates of the powdery mildew pathogen. Based on the identification of a specific *Pm3* haplotype and using molecular markers derived from the cloned *Pm3b* locus, additional known *Pm3* alleles (*Pm3a, Pm3b, Pm3c, Pm3d, Pm3e, Pm3f, Pm3g)* were isolated from different wheat lines (Srichumpa *et al*, 2005; Yahiaoui *et al*, 2006).

4. Conservation of genetic diversity in gene banks

Over the years, crop improvement has resulted in the depletion of genetic diversity of wheat. This is due to replacement of landraces and farmer's traditional cultivars with modern high yielding varieties. With the objective of preserving crop diversity, the worldwide gene banks conserve the wild crop relatives and traditional cultivars. These genetic collections possess a wide range of genetic diversity critical for enhancing and maintaining the yield potential, as they can provide new sources of resistance and tolerance to biotic and abiotic stresses

(Skovmand *et al*, 2002). There are more than 560,000 accessions of wheat held in gene banks around the world (Table 3. 2).

4.1 Allele mining in wheat and focused identification of germplasm

Genetic diversity held in gene banks remains largely unexplored at the molecular level due to a lack of fast and efficient tools to identify and study potentially useful new alleles. Still largely unexplored, a new approach of "allele mining" has recently become available and shows promise for the more efficient use of genetic diversity. It is a valuable approach which can provide insight into allelic variation of fully characterized genes of agronomic importance. Currently, there are attempts to apply this approach in important plant species such as maize, barley and rice (Latha *et al*, 2004; Stein *et al*, 2007; Harjes *et al*, 2008). There is an ongoing study in maize to identify alleles for *lcyE* gene and other genes that increase total carotenoids and that slow down the conversion of β-carotene to β-cryptoxanthin and zeaxanthin (Harjes *et al*, 2008). Barley germplasm is being screened to identify allelic variants of *Hv-eiF4E* which confers virus resistance (Stein *et al*, 2007). Latha *et al* (2004) reported the development of markers for allele mining of stress tolerance genes in rice germplasm. As the first wheat disease resistance genes have been cloned (Huang *et al*, 2003; Feuillet *et al*, 2003; Yahiaoui *et al*, 2004; Yahiaoui *et al*, 2006; Cloutier *et al*, 2007), the sequence information of these genes could allow the rapid analysis of the genetic diversity at these loci over a wide range of germplasm and also the subsequent identification of new alleles through allele mining.

Table 3.2: Global wheat collections (Source: The Global Crop Diversity trust, 2007)

Institute	No. of accessions	Country
CIMMYT, El Batan, Mexico	111,681	Global
ICARDA, Aleppo, Syria	37,830	Global
Agricultural Research Institute, Lushjne	6,000	Albania
Albanian Genebank, National Seed and Seedling Institute, Tirane	2,015	Albania
Banco Base Nacional de Germoplasma, Instituto de Recursos Biologicos,INTA	648	Argentina
AWCC, NSW, Department of Primary Industries, Tamworth	23,917	Australia
Agrobiology Seed Collection, Linz	876	Austria
Institute for Plant Genetic Resources "K. Malkov",Sadovo	9,747	Bulgaria
Recursos Geneticos e Biotecnologia, (EMBRAPA/CENARGEN), Brasilia	5,169	Brazil
Centro Nacional de Pesquisa de Trigo(CNPT;EMBRAPA), Passo Fundo	13,594	Brazil
Plant Gene Resources of Canada, Saskatoon	5,052	Canada
Institute of Crop Germplasm Resources (CAAS), Beijing	9,633	China
National Genebank (CYPARI), Agricultural Research Institute, Nicosia	7,696	Cyprus
Genebank Department**,** Research Institute for Crop Production, Prague	11,018	Czech Republic
Field Crops Institute, Agricultural Research Centre,Giza	2,867	Egypt
Plant Genetic Resources Centre, Institute of Biodiversity Conservation and Research, Addis Ababa	10,745	Ethiopia
Station d'Amelioration des Plantes, INRA, Clermont-Ferrand	14,200	France
Genebank, IPK, Gatersleben	9,633	Germany
Institute of Agrobotany, Tapioszele	7,531	Hungary
National Bureau of Plant Genetic Resources (NBPGR), New Delhi	32,880	India
National Genebank of Iran, Genetic Resources Division, Karaj	12,169	Iran
Lieberman Germplasm Bank, Institute of Cereal Crop Development, Tel-Aviv	5,500	Israel
Institute of Evolution, Haifa University, Haifa	1,000	Israel
Instituto del Germoplama, Bari	32,751	Italy
Genebank, National Institute of Agrobiological Sciences, Tsukuba	7,148	Japan
Plant Germplasm Institute, Graduate School of Agriculture, Kyoto	4,378	Japan
Centre for Genetic Resources (CGN, CPRO-DLO), Wageningen	5,529	Netherlands
PGRI, National Agricultural Research Centre, Islamabad	2,572	Pakistan
Plant Breeding and Acclimatisation Institute (IHAR), Radzikow	12,974	Poland
Banco de Germoplasma-Genetica, Estacao Agronomica Nacional, Oeiras	831	Portugal
Departmento de Genetica e Biotecnologia, Universidade Tras-os-Montes EAlto Douro,Vila Real	1,466	Portugal
Suceava Genebank, Suceava	1,543	Romania
N.I. Vavilov Research Institute of Plant Industry (VIR), St. Petersburg	39,880	Russia
Institute of Field and Vegetable Crops, Novi Sad	2,431	Serbia
Agricultural Research Council, Small Grains Institute, Bethlehem	2,527	S. Africa
Centro de Recursos Fitogeneticos, Madrid	3,183	Spain
Nordic Gene Bank, Alnarp	1,843	Sweden
Station Federale de Recherches en Production Vegetale de Changins, Nyon	6,996	Switzerland
Plant Genetic Resources Deptt, Aegean Agricultural Research Institute, Izmir	6,381	Turkey
Yurjev Institute of Plant Production, National Centre for Plant Genetic Resources of Ukraine, Kharkov	20,626	Ukraine
Crop Genetics Department, John Innes Centre, Norwich	9,584	UK
Wheat Genetic Resource Centre, Kansas State University, Manhattan, Kansas	5,000	USA
USDA/ARS, Wheat Genetic Stocks Collection, University of Missouri, Columbia, Missouri	3,000	USA
USDA/ARS, National Small Grains Research Facility, Aberdeen, Idaho	56,218	USA
44 institutes	562,831	**Total**

One major challenge to finding rare alleles from large collections is to identify a germplasm subset of a size that is economically feasible to screen, while maximizing the probability of finding the desirable trait. Core collections have been widely promoted as a means of approaching large collection using smaller subsets which represent maximum diversity. A new approach called Focused Identification of Germplasm Strategy (FIGS) was recently suggested (Mackay and Street, 2004) which can improve the efficiency of accessing the genetic resources. The central idea behind FIGS is to focus on the trait being sought and identify germplasm from collections sites where there would have been a selection pressure for the trait.

5. Aim of this thesis

The objectives of this study were:

(i) To screen the large set of 1320 landraces selected on the basis of eco-geographical criteria, for resistance to powdery mildew using a differential set of powdery mildew isolates and to characterize these resistant landraces for the presence of *Pm3*-like genes and for the presence of already known *Pm3* alleles.

(ii) To isolate allelic variants at the *Pm3* locus from a large subset of landraces and their functional assessment using virus induced gene silencing and transient transformation assay.

(iii) To determine sequence diversity at *Pm3* locus by isolating *Pm3* allelic sequences from a set of tetraploid wheat species, their comparison to hexaploid wheat *Pm3* sequences and to analyse the evolution of this locus.

The large set of landraces was established through focused identification of germplasm strategy (FIGS) in order to maximize the chances for identification of powdery mildew resistance sources. The final set of 1320 landraces was screened for powdery mildew resistance using four *Bgt* isolates which led to identification of 211 resistant or intermediately resistant landraces to at least one of the four isolates tested. These 211 landraces were molecularly analysed for the presence of *Pm3*-like genes, and also for the presence of known *Pm3* alleles. Out of the 211 lines (from 1320), 145 showed the presence of a *Pm3* haplotype and the following search for the seven known *Pm3* resistance alleles in these 145 lines revealed the presence of *Pm3b* and *Pm3c* in 30 and 4 lines, respectively. This analysis led to identification of the landraces which served as the candidates for isolation of new *Pm3* alleles. Sixteen new *Pm3* alleles were isolated, out of which seven functionally active alleles *Pm3l, Pm3m, Pm3n, Pm3o, Pm3p, Pm3q* and *Pm3r* extend the previously known series of *Pm3* alleles. We also found that at least two of the new alleles isolated from landraces confer slow resistance which indicate that this might be the reason why these alleles were not selected in breeding germplasm.

We have also screened a collection of wild and domesticated tetraploid wheat lines for *Pm3* genes and studied their genetic differentiation. We found that the evolution of functional *Pm3* resistance genes occurred independently in the tetraploid and hexaploid wheat species.

IV. Molecular approaches for characterization and use of natural disease resistance in wheat

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1. Abstract

Wheat production is threatened by a constantly changing population of pathogen species and races. Given the rapid ability of many pathogens to overcome genetic resistance, the identification and practical implementation of new sources of resistance is essential. Landraces and wild relatives of wheat have played an important role as genetic resources for the improvement of disease resistance. The use of molecular approaches, particularly molecular markers, has allowed better characterization of the genetic diversity in wheat germplasm. In addition, the molecular cloning of major resistance (*R*) genes has recently been achieved in the large, polyploid wheat genome. For the first time this allows the study and analysis of the genetic variability of wheat *R* loci at the molecular level and therefore, to screen for allelic variation at such loci in the gene pool. Thus, strategies such as allele mining and ecotilling are now possible for characterization of wheat disease resistance. Here, we discuss the approaches, resources and potential tools to characterize and utilize the naturally occurring resistance diversity in wheat. We also report a first step in allele mining, where we characterize the occurrence of known resistance alleles at the wheat *Pm3* powdery mildew resistance locus in a set of 1320 landraces which was assembled on the basis of eco-geographical criteria. From the known *Pm3 R* alleles, *Pm3b* was the only one frequently identified (3% of the tested accessions). In the same set of landraces, we found a high frequency of a *Pm3* haplotype carrying a susceptible allele of *Pm3*. This analysis allowed the identification of a set of resistant lines where new potentially

functional alleles would be present. Newly identified resistance alleles will enrich the genetic basis of resistance in breeding programs and contribute to wheat improvement.

2. Introduction

Wheat is globally one of the three most important food crops, the other two being maize and rice. Wheat diseases cause severe yield losses and often reduce grain quality. Some of the most important fungal diseases of wheat include three rust species (stripe, leaf and stem rust), powdery mildew, fusarium, septoria, mycosphaerella and tan spot. To achieve sufficiently high resistance to fungal pathogens is an ongoing challenge for wheat breeding. Various aspects such as understanding pathogen biology, characterization of pathogen avirulence genes as well as of plant disease resistance genes, and finally the search for new resistance sources, all contribute to develop wheat with increased resistance to various diseases.

Wheat (*Triticum aestivum*) is an allopolyploid species featuring three distinct, homoeologous genomes A, B and D. As the wheat genome is large (16×10^9 bp) and the major fraction consists of repetitive sequences, thus, the molecular cloning of genes, for which only genetic information is available, remains a challenge (Keller *et al*, 2005). Therefore, the first efforts to characterize loci of disease resistance at the molecular level concentrated on the development of molecular markers linked to these important traits.

The availability of molecular markers linked to specific resistance genes and of information on their genetic location has supported resistance breeding by simplifying the detection of specific genes in breeding material. This makes the selection process faster and more cost effective. In addition, different genes can be combined in a pyramiding strategy for resistance breeding. Despite these efforts, the genetic base of

disease resistance in wheat remains dangerously narrow and adaptation of pathogens is always a threat, challenging the resistance of existing elite material. Currently, the emergence and spread of the new virulent stem rust race *Ug99* is considered to be a potential threat to wheat production worldwide. The *Ug99* race has overcome the major stem rust resistance gene *Sr31* (http://www.ars.usda.gov/Main/docs.htm?docid=14649). Previously, there was no report on virulence against *Sr31*, a gene which is widely used in India, China, Europe and South America. This makes it imperative to identify new sources of resistance that might exist in germplasm collections and then to make combinations with existing sources to develop more durable types of resistance.

The introduction of resistance genes from landraces, traditional varieties and wild relatives, e.g. the diploid and tetraploid progenitors of hexaploid wheat, has been successful to broaden resistance *(*Miranda *et al*, 2006; Liu *et al*, 2002; Rong *et al*, 2000). Over thousands of years, landraces of hexaploid wheat have developed under a variety of different edaphic and climatic environments. This has resulted in the evolution of a large number of ecotypes adapted to specific local environments. Thus, the genetic collections available in gene banks are expected to provide a rich resource to identify new functional genes or alleles of resistance genes. The molecular changes underlying this adaptation are mostly unknown and this diversity is largely unused and uncharacterized at the molecular level. Traditionally, resistance genes in wild relatives of wheat have been introgressed by complex breeding schemes involving irradiation and chromosomal translocations (Baum *et al*, 1992). This resulted in the introgression of large chromosomal segments, often carrying negative breeding traits (linkage drag). The molecular isolation of the underlying genes and their use through transgenic technologies will contribute to an efficient future use of resistance genes from wild grasses.

Hybridization and introgression of chromosomal segments, marker assisted selection and the breeding of synthetic hexaploid wheat (Zhang *et al,* 2005) are well established methods to broaden the genetic diversity of disease resistance in wheat. Still largely unexplored, a new approach of "allele mining" has recently become available and shows promise for the more efficient use of genetic diversity (to be discussed in detail later in this paper). The first wheat disease resistance genes have been cloned at the molecular level (Huang *et al,* 2003; Feuillet *et al,* 2003; Yahiaoui *et al,* 2004; Srichumpa *et al,* 2005; Yahiaoui *et al,* 2006; Cloutier *et al*, 2007). The sequence information of these cloned genes facilitates the rapid analysis of the genetic diversity at these loci over a wide range of germplasm and the subsequent identification of new alleles through allele mining. Molecular tools that specifically access the existing genetic diversity at a particular locus provide a promising approach to utilize the diversity maintained in the gene banks globally. In this paper, we discuss the molecular approaches available to detect and use genetic diversity for improving disease resistance in wheat. We also describe an allele mining strategy for new resistance specificities at the wheat resistance locus *Pm3* against the powdery mildew pathogen (*Blumeria graminis* f.sp. *tritici*) that was applied to 1320 wheat landraces from different geographic origin.

3. Molecular markers in the characterization of wheat disease resistance diversity

Molecular markers play a significant role in the process of identification and introgression of natural resistance into economically important but susceptible breeding material. The range of molecular markers used for this purpose includes restriction

fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), sequence tagged sites (STS), simple sequence repeats (SSR)/ microsatellites, expressed sequence tags (ESTs) and RGAs (resistance gene analogs). RFLPs were the first molecular markers to be used in wheat in the early 90's. There are several reports on the essential role of RFLPs in marker assisted selection, genome mapping as well as characterization and isolation of various disease resistance genes in wheat (Lagudah *et al*, 2006; Feuillet *et al*, 2003). Because of lower costs and time requirements, PCR based markers have a higher potential for applications in the genetic characterization of wheat germplasm than RFLPs. AFLPs combine the simplicity of the RAPDs and robustness of RFLPs. Therefore, they have been frequently used to investigate biodiversity in several crops. Diagnostic markers for different resistance genes in wheat have been developed by using the AFLP technique (William *et al*, 2003; Adhikari *et al*, 2003).

STS markers have been highly useful tools for the screening of natural genetic variation as well as for tagging of resistance genes and QTLs in wheat (Lagudah *et al*, 2006; Tyryshkin *et al*, 2006; Liu *et al*, 2002). Given the vast molecular information available on various plant disease resistance genes, RGAs (resistance gene analogs) have been extensively used. For instance, RGA markers linked to resistance genes *Yr5, Pm21* and *Pm31* (Yan *et al*, 2003, Chen *et al*, 2006, Xie *et al*, 2004) have been identified. As RGA sequences are very common in the plant genomes, they can be considered as a type of random markers. SSR/microsatellite markers are highly polymorphic in wheat and are now widely used in wheat genetics for diversity studies, genotype identification (Prasad *et al*, 2000), marker-assisted selection, mapping, identification and tagging of disease resistance genes (Chagué *et al*, 1999; Miranda *et al*, 2006; Liu *et al*, 2002; Adhikari *et al*, 2003). Recently, microsatellite markers have been found linked to leaf

rust resistance gene *Lr34* (Bossolini *et al*, 2006) supporting the identification of wheat genotypes with *Lr34*. With the increasing availability of molecular maps based on SSR markers, the identification and cloning of important genes may become more straightforward. Multiallelism, chromosome-specificity, even distribution in the genome and the possibility of high-throughput fingerprinting of large number of accessions are the properties that make SSR markers a good choice for detection of genetic polymorphism and diversity among wheat lines. However, microsatellite markers are often not suitable to define homoeologous loci, thus limiting their use in intraspecific and intragenomic studies (Gupta *et al*, 1999). This also complicates the use of SSR markers for introgression studies involving wild relatives of wheat. Table 4.1 gives a summary of some molecular markers currently used for tagging different disease resistance genes in wheat.

Table 4.1: Examples of molecular markers used for characterization and genetic mapping of different disease resistance genes in wheat.

Disease	**Resistance gene/genomic region**	**Marker used for identification and/or mapping**	**Reference**
Leaf rust, yellow rust and stem rust	*Lr34/Yr18*	SSR, STS	Bossolini *et al*, 2006 Lagudah *et al*, 2006
	Lr9, *Lr19* and *Lr24*	SSR	Chagué *et al*, 1999
	Lr46	AFLP	William *et al*, 2003
	Lr47	RFLP/CAPS	Helguera *et al*, 2000
	Yr15	STS	Tyryshkin *et al*, 2006
	Yr5	RGA	Yan *et al*, 2003
	Yr10	RGA	Bariana *et al*, 2001
	Sr30	AFLP, RFLP	Bariana *et al*, 2001
	Sr36	SSR	Bariana *et al*, 2001
Powdery mildew	*Pm3*	STS	Tommasini *et al*, 2006
	Pm21	RGA	Chen *et al*, 2006
	Pm26	RFLP	Rong *et al*, 2000
	Pm30	SSR	Liu *et al*, 2002
	Pm31	RGA	Xie *et al*, 2004
	Pm34	SSRs	Miranda *et al*, 2006
Septoria leaf blotch	*Stb8*	AFLP and SSRs	Adhikari *et al*, 2003

4. Map based cloning of disease resistance genes in wheat

To design precise and targeted molecular tools for diversity analysis based on allele mining, knowledge on the DNA sequence at a particular resistance locus becomes important, requiring the molecular cloning of genes. DNA sequence information is essential to devise rapid and inexpensive PCR strategies to isolate alleles of identified resistance genes from a wide range of cultivars, landraces and related species.

In map-based cloning, saturation of the genomic region of interest is greatly supported by genomic information and markers obtained from the grass model species rice and brachypodium (Griffiths *et al*, 2006). In addition, there are now a number of BAC libraries available from diploid, tetraploid and hexaploid wheat species (Keller *et al*, 2005) supporting map-based cloning strategies of wheat genes. Until now, three leaf rust resistance genes (*Lr*) and one allelic series of a powdery mildew (*Pm*) resistance gene have been cloned from wheat: *Lr21* (Huang *et al*, 2003), *Lr10* (Feuillet *et al*, 2003), *Lr1* (Cloutier *et al*, 2007) and the *Pm3* alleles (Yahiaoui *et al*, 2004; Srichumpa *et al*, 2005; Yahiaoui *et al*, 2006).

The first cloned wheat disease resistance gene, *Lr21*, was incorporated into bread wheat cultivar Thatcher from the diploid wheat ancestor *Ae. tauschii*. A diploid/ polyploid shuttle mapping strategy was deployed for map based cloning of *Lr21* (Huang *et al*, 2003). There, the genetic analysis was done in hexaploid wheat but the large insert cosmid library was developed from the diploid donor. *Lr21* was chosen for cloning because of its location in a gene rich region and of the extensive allelic diversity at this locus in natural populations of *Ae. tauschii* (Huang & Gill, 2001).

Feuillet *et al*, (2003) isolated the leaf rust resistance gene *Lr10*, located on chromosome 1AS in hexaploid wheat by combining subgenome map-based cloning (Stein *et al*,

2000) and haplotype studies in the genus *Triticum*. The chromosome walking was done on BAC clones of the diploid wheat *T. monococcum* DV92 (A genome) which had an *Lr10* haplotype while the genetic map was constructed on the basis of genetic data from a hexaploid wheat population segregating for the *Lr10* resistance.

The wheat powdery mildew resistance gene *Pm3,* a dominant gene on chromosome 1AS, exists in 10 different alleles (*Pm3a-Pm3j*) as identified by classical genetic studies. These alleles are predicted to confer resistance to specific races or isolates of the powdery mildew pathogen. Yahiaoui *et al,* (2004) used the combined analysis of genomes from wheat species with different ploidy levels for positional cloning of the *Pm3b* allele of *Pm3* in bread wheat. This represented the first molecular isolation of a powdery mildew resistance gene from wheat and a breakthrough for further analysis of diversity and evolution of this important locus. Based on the identification of a specific *Pm3* haplotype and using molecular markers derived from the *Pm3b* locus, additional known *Pm3* alleles (*Pm3a, Pm3c, Pm3d, Pm3e, Pm3f, Pm3g)* were isolated from different wheat lines (Srichumpa *et al,* 2005; Yahiaoui *et al,* 2006). Interestingly, it was also found that the three alleles *Pm3h*, *Pm3i*, *Pm3j* are actually identical to *Pm3d*, *Pm3c* and *Pm3b* respectively (Yahiaoui *et al*, 2006), suggesting that the lines in which the *h* to *j* alleles were identified contain additional resistance genes.

Quantitative resistance is often assumed to be more durable than single-gene resistance. Therefore, the improvement of quantitative resistance through tagging and cloning of QTLs is of increasing importance in several wheat research and breeding programs. A few QTL have been cloned in plants mainly by positional cloning (Salvi & Tuberosa, 2005). In wheat, no QTLs for disease resistance have yet been cloned but there are many ongoing projects with this goal. These include projects aiming at the isolation of rust resistance loci such as *Lr34/Yr18* (Bossolini *et al*, 2006; Lagudah *et al,* 2006;

Spielmeyer *et al*, 2005) and *Lr46/Yr29* (William *et al*, 2003; Rosewarne *et al*, 2006), *Sr2* (Kota *et al*, 2006) and a major QTL for Fusarium head blight resistance (Liu & Anderson, 2003). Mardi *et al* (2005) reported the tagging of QTL responsible for Fusarium head blight (FHB) resistance with SSR markers and suggested that the SSR markers linked to the QTL would facilitate marker-assisted selection for FHB resistance in wheat. QTL tagging and cloning provide tools to the breeder for marker assisted selection of complex disease resistance traits. It should also help to understand the respective roles of specific resistance loci versus partial resistance genes and the interactions between the genes and the environment.

5. Genetic resources for improvement of wheat disease resistance

A big advantage of diversity studies in wheat, compared to model plants such as Arabidopsis, is the existence of large collections of wild and cultivated diploid, tetraploid and hexaploid species secured in gene banks. However, at the molecular level this diversity remains largely unexplored due to a lack of fast and efficient tools to identify and study potentially useful new alleles. In addition to wheat landraces, wild relatives of wheat have been always explored and exploited as sources of new resistance genes. For example, a number of *R* genes originate from wild wheat relatives: the stem rust resistance gene *Sr39* was transferred from the wild relative *Aegilops speltoides* L. to bread wheat cultivar Thatcher, leaf rust resistance gene *Lr24* from *Agropyron elongatum, Lr47* and *Pm32* from *Aegilops speltoides*, *Pm6* from *T. timopheevi* (Allard & Shands, 1954), *Pm26* and *Pm30* from *Triticum turgidum var dicoccoides* and *Pm34* from *Aegilops tauschii Coss.* (Miranda *et al,* 2006; Liu *et al,* 2002; Rong *et al,* 2000). Common wheat has also been genetically improved for many

decades through the introgression of rye chromatin. The rye chromosome arm 1RS is the most widely incorporated alien variation in the wheat genome. To give an example, wheat cultivar *Amigo* carries the powdery mildew resistance gene *Pm17* on its introgressed 1RS chromosome arm (Forsström & Merker, 2001).

As each of these *R* genes usually act only against a subset of the existing pathogen races, combinations of genes as well as the identification of new resistance genes/alleles are essential. Identification of new resistance genes or of new alleles at already known loci is classically done by infection experiments on landraces or wild relatives of wheat followed by crosses necessary to determine if the resistance is due to a single gene and if the gene is a new allele at a known locus or represents a new locus. As resistance may be lost with rapid emergence of new pathogen strains, it becomes a continuous task to identify new resistance genes and to transfer these genes into common wheat if they are present in wild relatives or related species.

6. Molecular tools for screening the diverse germplasm: Allele mining in cereals

The development of molecular tools to specifically access the existing genetic diversity at particular loci facilitates the rapid analysis of allelic diversity in the gene pool of wheat and its relatives. This in turn allows the molecular isolation of new alleles with potential agronomical relevance and a more efficient and targeted use of genetic resources for research and breeding. The strategy of finding valuable, unknown alleles at a known locus is referred to as ‘allele mining’. In allele mining, the sequence of a target gene is used to develop specific markers to amplify, isolate and sequence new alleles at that particular locus. It seems to be a promising, although largely untested

method to unlock the diversity in the collections of genetic resources in the world genebanks.

There are reports about the allele mining strategy in several cereal species to isolate alleles of target genes. In barley, an evaluation of cultivated germplasm was carried out to detect the presence of thermostable alleles of β-amylase (*Bmy-Sd2H* and *Bmy-Sd3* alleles) that improves the fermentability during brewing (Malysheva *et al*, 2004). The study was carried out on 891 accessions originating from different geographic regions worldwide. This led to the identification of 166 accessions with superior alleles, suggesting that the improvement of malting quality in barley could be achieved by introducing these alleles into breeding programs. Latha *et al*, (2004) used the rice calmodulin genes and a salt inducible rice gene for allele mining of stress tolerance genes on identified accessions of rice and related germplasm. They examined the feasibility of allele mining using PCR primers based on the 5'- and 3'-untranslated regions of genes and found that these primers were sufficiently conserved to be effective over the entire range of germplasm in rice. The new HMW-glutenin alleles encoded by the *Glu-R1* locus of *Secale cereale* (rye) have been analysed and characterized (De Bustos & Jouve, 2003) from different rye cultivars and their most closely related wild subspecies. Primers designed from a nucleotide sequence of the allele *Glu-Dly10*, which recognised the upstream and downstream flanking positions of the coding regions of the genes, were used in the study. Thus, allele mining supports the discovery of new alleles of target genes. However, the limitation of this approach in wheat lies in the fact that, very few genes of agronomical importance have yet been cloned. This is particularly true for genes involved in disease resistance.

Ecotilling (Comai *et al*, 2004) represents a specific approach to allele-mining and refers to a high throughput screening technique for the discovery of polymorphisms in natural

populations. It can serve as a cheaper alternative to full DNA sequencing when searching for rare polymorphism, but similar to the other allele-mining strategies it still requires specific sequence information for the target gene. Ecotilling can also be used for mapping, association analysis, mutational profiling and biodiversity studies. It has been successfully used in Arabidopsis (Comai *et al*, 2004) where 55 haplotypes of 5 genes have been discovered after screening of more than 150 individuals. The discovered polymorphisms were confirmed by sequencing and base pairs changes, insertions, deletions and variation in microsatellite number were observed.

7. Focused Identification of Germplasm Strategy (FIGS) and allele-mining for molecular diversity at the *Pm3* locus

To test a strategy of allele mining in wheat using a large set of diverse germplasm, we focused on the *Pm3* resistance locus as there is extensive sequence information available for targeted allele cloning. A subset of bread wheat landraces were selected for the study using the FIGS (Focused Identification of Germplasm Strategy) system (Mackay, Street *et al*, manuscript in preparation. Also see www.figstraitmine.com). In this case, the eco-geographic profile of 400 accessions, from the USDA-ARS National Small Grains Collection, with known powdery mildew resistance was identified. This profile was then used as a template to identify environmentally similar collection sites from the FIGS database of nearly 17,000 landraces. Individual accessions were selected using multivariate statistical procedures that determined how eco-geographically similar the collection site of a given accession was to the resistant set template. The FIGS Powdery Mildew Set of accessions finally includes 899 landraces from ICARDA (International Centre for Agricultural Research in the Dry Areas, Syria), 295 landraces

from AWCC (Australian Winter Cereals Collection) and 126 landraces from VIR (N.I. Vavilov Research Institute of Plant Industry, Russia), making a total of 1320 landraces. These originate from Turkey (419), Iran (391), Afghanistan (292), Pakistan (133), Armenia (34), Turkmenistan (16), Russia (9), India (6), Azerbaijan (1) and Uzbekistan (1).

8. Screening and identification of powdery mildew resistant lines

For characterization of the "FIGS powdery mildew set" we used a combined strategy of screening for genetic diversity with molecular markers and classical pathogenicity tests. The entire 'FIGS powdery mildew set' was screened with a differential set of powdery mildew isolates to select a subset of resistant landraces for molecular analysis. The detached leaf segments from seven day old plants were placed on phytagar media and subjected to infection with four different isolates of powdery mildew (Figure 4.1). The choice of the isolates was based on the pattern of their avirulence/virulence to the known alleles of *Pm3*. The four isolates used were 96224, 98275 and 96244 (avirulent on most known *Pm3* alleles) and 2000.15.Syros (virulent on all the known *Pm3* alleles). The phenotypes were grouped in three categories: resistant (R), intermediate (I) and susceptible lines (S). The scoring was done after 9-10 days of infection, on a 1 to 100% susceptibility scale, i.e. the leaf area covered with mildew was ranked phenotypically where lines with 100% leaf area covered with mildew were considered fully susceptible while 0% marks complete resistance. This screening led to the selection of 211 resistant or intermediate resistant lines to at least one of the four mildew isolates used in the screen.

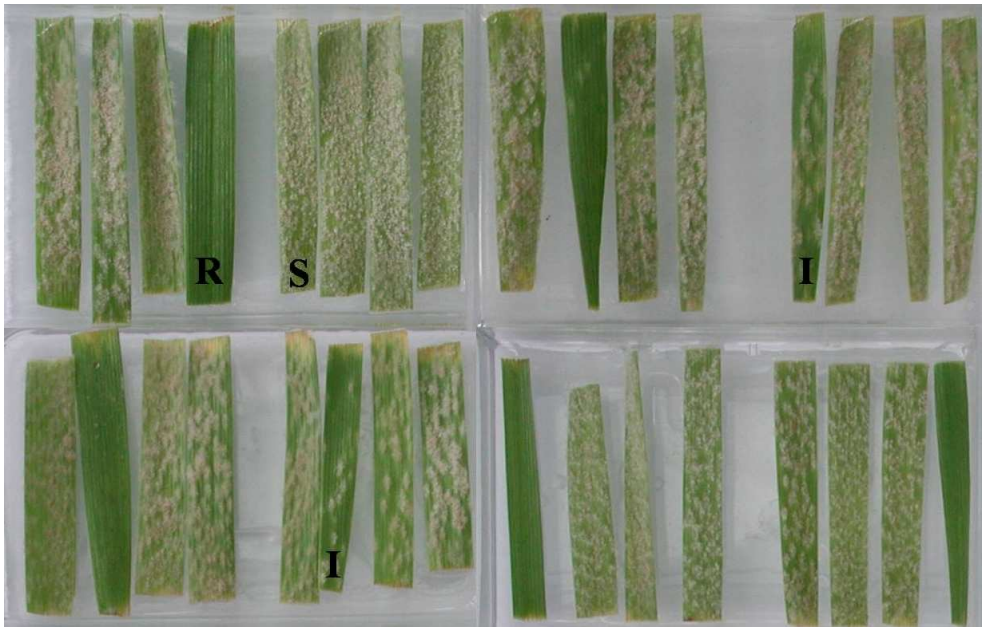

Figure 4.1: Phenotypic assay of wheat landraces for powdery mildew resistance by infection with powdery mildew isolate 96244.
Powdery mildew resistant (R), Intermediate (I) and susceptible leaves (S) are marked.

9. PCR based approach for characterization of *Pm3* alleles

We first tested the molecular tools available for the detection of the *Pm3* gene in a subset of 295 AWCC landraces. We used an STS marker obtained from haplotype studies at the *Pm3* locus (Yahiaoui *et al,* 2004; Srichumpa *et al,* 2005). This *Pm3* haplotype marker amplifies a 946bp fragment originating from the 5' non-coding region of *Pm3b* which is diagnostic for the presence of a *Pm3* gene (Figure 4.2). The *Pm3* haplotype was present at an unexpectedly high frequency in the subset of the 'FIGS powdery mildew set' tested. In the 295 AWCC landraces, amplification of the *Pm3* STS marker was found in 257 lines (87.1%). This high percentage prompted us to check this subset for the presence of the already known alleles (*Pm3a-Pm3g*) using *Pm3* allele specific markers. These markers were developed in our lab (Tommasini *et al,* 2006) based on the specific nucleotide polymorphisms of coding and adjacent non-coding regions of each of the *Pm3* alleles.

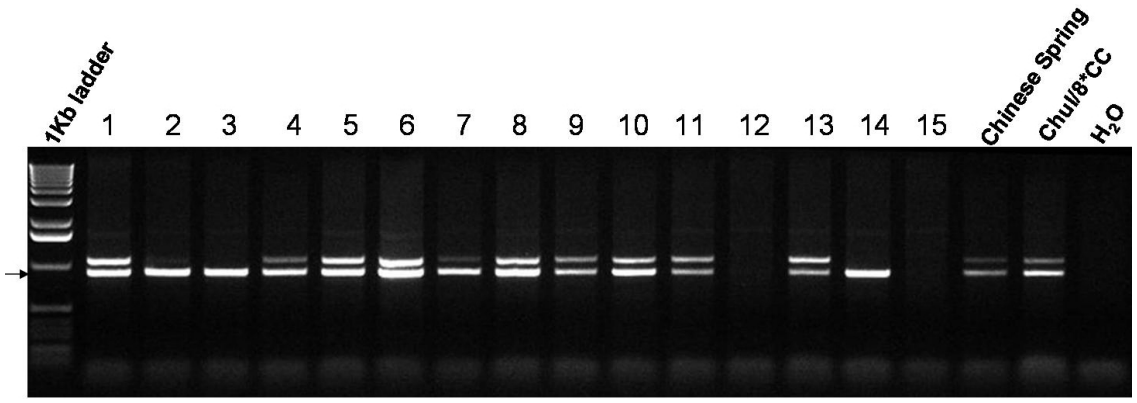

Figure 4.2: PCR amplification in the "FIGS powdery mildew set" of the STS marker specific for the *Pm3* haplotype.

The lower band (see arrow) corresponding to chromosome 1A is diagnostic for the presence of a *Pm*3 like gene. The numbers 1-15 correspond to the tested landraces, Chinese Spring & Chul/8*CC are the positive controls while H_2O serves as the negative control.

We found that the *Pm3b* allele was the only known functional *Pm3* allele present in the subset. It was detected in seven lines. This demonstrated that most of the alleles of *Pm3* in the subset do not correspond to known resistance alleles. The infection data obtained from the powdery mildew infection described above showed that only 40 out of 295 lines were resistant or intermediate resistant to at least one of the isolates while the other 255 lines were susceptible to the tested isolates. This indicated that susceptible alleles of *Pm3* are present in at least 86.4% of the lines and are therefore expected to be widespread among the landraces. This percentage is possibly even higher, given the fact that resistance to powdery mildew might not be due to a gene at the *Pm3* locus but may be caused by any of the known or still uncharacterized resistance genes in the germplasm. Therefore, in the particular case of *Pm3* allele mining, the strategy of screening the lines with different mildew isolates prior to sequencing was chosen.

However, for other genes and traits sequencing the complete set of germplasm without prior phenotypic analysis might be a valid alternative strategy.

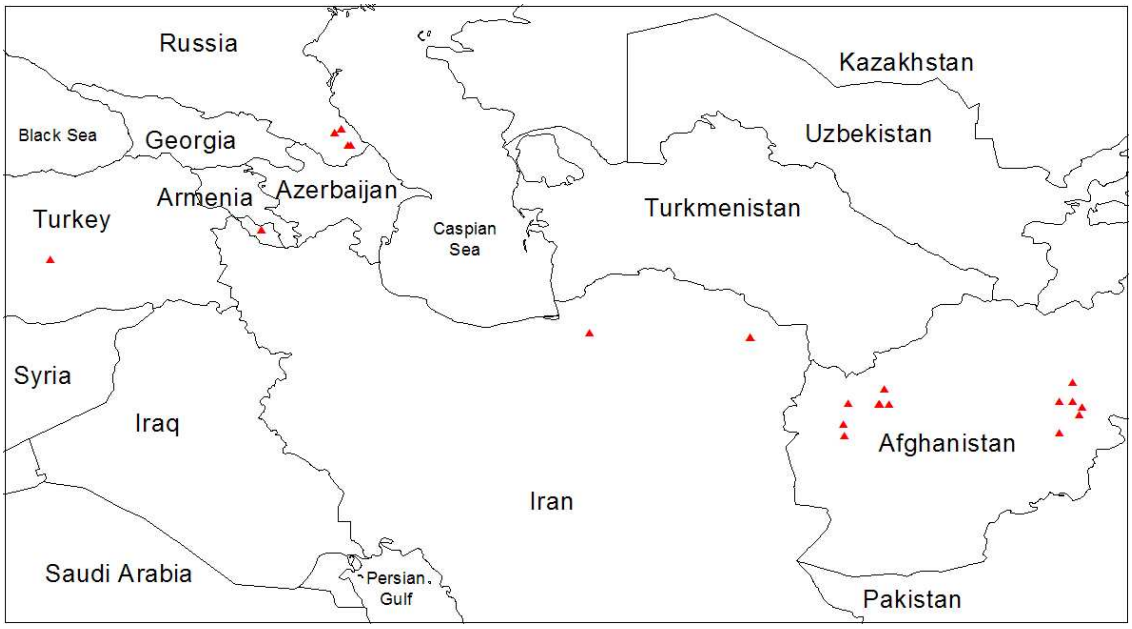

Figure 4.3: Geographic origin of the 30 *Pm3b* lines detected in the 'FIGS powdery mildew set' of 1320 landraces.
The collection sites are indicated by red triangles.

The 211 intermediate or resistant lines selected during the infection screen were subjected to molecular analysis for the *Pm3* locus. Out of the 211 lines (from 1320), 145 showed the presence of a *Pm3* haplotype. The search for the seven known *Pm3* resistance alleles in the 145 lines revealed the presence of *Pm3b* and *Pm3c* in 30 and 4 lines, respectively. Thus, *Pm3b* was the most frequent *Pm3* allele in the landrace set. It was identified in landraces originating from Afghanistan (15), Iran (6), Russia (6), Azerbaijan (2) and Turkey (1), while the 4 landraces with *Pm3c* allele originated from Iran (3) and Azerbaijan (1) [see Table 4.2 for a summary of results and Figure 4.3 for geographic distribution of *Pm3b* lines]. The first identification of the *Pm3b* allele was in a landrace from Uzbekistan (http://www.ars-grin.gov/npgs/index.html), which is

consistent with its frequency and actual geographical distribution particularly in Afghanistan, a neighbouring country to Uzbekistan (Figure 4.3).

Table 4.2: *Pm3* allele mining: the frequency of known *Pm3* resistance alleles found in the 'FIGS powdery mildew set' of 1320 lines is presented.

Detection was done with the *Pm3* haplotype specific and *Pm3* allele-specific primers.

Specific PCR	**Number of lines tested for *Pm3* haplotype and known *Pm3* alleles**	**Number of lines possessing *Pm3* haplotype and *Pm3* known alleles**	**Landrace**	**Origin**
Pm3 haplotype	211	145		
Pm3a, Pm3d, Pm3e, Pmf, Pm3g	145	0		
Pm3b	145	15	AUS9943, AUS9948, AUS10003, AUS10033, AUS13239, AUS13297, AUS13306, AUS13307, AUS13311, AUS14504, AUS14532, AUS14840, VIR 45538, VIR 49005, VIR 49006	Afghanistan
		6	IG 122348, IG 122354, IG 122361, IG 122373 IG 122502, VIR 38613	Iran
		2	VIR 16766, VIR 31595	Azerbaijan
		6	VIR 23918, VIR 23922 VIR 34986, VIR 35021 VIR 35030, VIR 34984	Russia
		1	VIR 35203	Turkey
Pm3c	145	3	IG 122491, IG 122372 IG 122346	Iran
		1	VIR 46301	Azerbaijan

To summarize, the strategy for the identification of new *Pm3* alleles described in this paper included phenotypic screening of the "FIGS powdery mildew set" of landraces for powdery mildew resistance, molecular analysis of *Pm3* haplotype composition and determination of known *Pm3* resistance alleles. This resulted in 111 candidate lines (9% of total set) to specifically target for further characterization of the gene present at the *Pm3* locus. These candidate lines (i) are resistant or intermediate resistant to at least one of the isolates tested (ii) possess the *Pm3* haplotype and (iii) lack any of the known *Pm3* alleles (145-34= 111). It is expected to find new *Pm3* resistance alleles among these lines, although the presence of susceptible *Pm3* alleles cannot be ruled out (based on the results of AWCC subset, presented earlier in this paper). The resistant phenotype in such cases may be attributed to the presence of other *Pm* genes.

The approach described above is one of the first large-scale attempts of a systematic resistance allele-mining from wheat landraces using molecular tools derived from the target gene sequence. Putative new *Pm3* resistance genes will be functionally tested by using a transient transformation assay (Yahiaoui *et al*, 2004) and other approaches such as virus induced gene silencing (VIGS, Scofield *et al*, 2005). This should reveal whether the newly isolated genes are actually active resistance alleles and if they confer new resistance specificities to the plant. Finally, the newly identified *Pm3* alleles could be transferred by classical genetic crosses to powdery mildew susceptible cultivars or alternatively be transformed into susceptible varieties as single genes. In addition, they could be combined as *R* gene cassettes to achieve a disease control which is possibly more durable. Besides these more applied aspects in wheat breeding, the analysis of allelic diversity and accumulation of diverse allelic sequences will contribute to a better characterization of the mechanisms involved in resistance gene evolution. The identification of new functional *Pm3* alleles from diverse germplasm will also

contribute to the molecular understanding of *R* gene function. The comparison of sequences from new alleles can clarify the molecular basis of *Pm3* specificity, e.g. by studying chimeric genes created by domain swap experiments with domains from the newly identified sequences.

10. Concluding remarks

A more efficient exploitation of the genetic diversity in gene banks is essential for meeting the challenges that wheat breeding is facing in the decades to come. However, the use of this diversity is hampered by the sheer number of accessions available and the limited resources which are at hand for phenotypic characterization of all these lines. Therefore, it is necessary to (i) develop strategies to assemble focused sets of material for specific traits based on rational criteria for selection of the lines but also (ii) to identify genes underlying agronomically important traits and (iii) establish the molecular tools for rapid characterization of new alleles.

V. Unlocking genetic resources for disease resistance in wheat landraces: molecular identification of new functional alleles at the *Pm3* locus.

Navreet Kaur, Kenneth Street, Michael Mackay, Nabila Yahiaoui and Beat Keller

1. Abstract

Landraces and wild relatives have traditionally been a rich source of genetic variation used in plant breeding. Given the increasing need for improved crop varieties with better agronomic properties, new strategies for a more efficient use of existing, but uncharacterized, genetic diversity is essential. Here, we have identified naturally occurring alleles of the powdery mildew resistance gene *Pm3* from a large set of 1320 bread wheat landraces stored in gene banks. The set of landraces was established by focused identification of germplasm from a database of nearly 17'000 landraces. Based on a hierarchical selection procedure, 16 new *Pm3* sequences were isolated. The sequence variation among the 16 new *Pm3* alleles and in comparison to the known *Pm3* alleles occurs mainly in the leucine rich repeat domain. Half of the new alleles were derived from Turkish landraces, while the other alleles originated from Afghanistan, Turkmenistan, Azerbaijan and Pakistan. Seven of the new alleles conferred resistance against powdery mildew in a transient transformation assay. At least, two of them have a novel specificity compared to the already known alleles of *Pm3*. The tested alleles from landraces conferred slow responses resulting in a late hypersensitive response with extensive tissue damage. These data suggest that the *Pm3* alleles in elite wheat lines were preferentially selected for their rapid and complete action. The study presented here is an example of efficient and targeted utilization of genetic resources by allele mining and reveals wheat landraces as a rich resource of new functional genes.

2. Introduction

Wheat is one of the most important food crops for humans. To provide sufficient food for the growing population of the world there is a strong need to increase crop production (Hoisington *et al*, 1999). Pathogens threaten wheat production by causing major yield losses. Control of diseases by chemicals is expensive and can have negative impacts on natural ecosystems whereas resistance genes offer efficient control and provide yield stability. Natural biodiversity has been used as a rich resource for improving the genetic basis of cultivated plants with novel alleles that improve productivity and adaptation (Gur and Zamir, 2004). In tomato, lines with introgression of wild alleles from *Lycopersicon hirsutum* have been created, which outperform the original elite variety by 48% and 33% for yield and fruit colour, respectively (Bernacchi *et al*, 1998). The *mlo* resistance allele *mlo-11* which provides resistance against barley powdery mildew originates from Ethiopian landraces (Piffanelli *et al*, 2004).

During domestication and further agricultural development, wild species have been replaced by landraces and traditional varieties, which were later again substituted by genetically less diverse modern cultivars. Thus, domestication, modern breeding and selection within adapted gene pools have imposed genetic bottlenecks with possible loss of valuable alleles in the breeding germplasm (Tanksley and McCouch, 1997). Therefore, seed bank collections are essential to conserve plant biodiversity and the alleles needed for resistance and tolerance to diseases, pests and harsh environments, and thus, pay big dividends to agriculture (Johnson, 2008). Thus, the favorable alleles lying unattended and uncharacterized in the gene banks could be of great value for crop improvement (Tanksley and McCouch, 1997).

Despite some studies that illustrate the utilization of genetic resource collections to find new sources of resistance (Yu *et al*, 2008; Singrun *et al*, 2004), the globally available large germplasm collections are still underutilized genetically. The major challenge to identify rare alleles from

large collections is to identify a germplasm subset with a size that is economically feasible to screen, while maximizing the probability of finding the desired trait. Core collections have been widely promoted as a means of approaching large collection using smaller subsets that represent maximum diversity. Focused Identification of Germplasm Strategy (FIGS) was recently suggested (Mackay and Street, 2004) as a rational and effective approach to access large collections of germplasm when looking for specific adaptive traits. The FIGS exploits the fact that a genotype is a product of selection pressure and evolutionary processes. In other words, when we know where an ex-situ accession evolved, or has had a long history of cultivation, we can predict what sorts of selection pressures may have been acted on the genotype and thereby what traits the phenotype is likely to express.

Genetic variation is often linked to allelic diversity at the relevant loci. A relatively new and underexplored, yet promising method to identify new alleles at a known locus is 'allele mining'. It is a valuable approach which can provide insight into allelic variation of fully characterized genes of agronomic importance. Currently, there are attempts to use this approach in important plant species such as maize, barley and rice (Harjes *et al*, 2008; Stein *et al*, 2007; Latha *et al*, 2004). There is an ongoing study in maize to identify alleles for the *lcyE* gene and other genes that increase total carotenoid content and that slow down the conversion of β-carotene to β-cryptoxanthin and zeaxanthin (Harjes *et al*, 2008). Allele mining is also used for identifying alleles at genetic loci involved in biotic and abiotic resistance. Barley germplasm is being screened to identify allelic variants of *Hv-eIF4E* for virus resistance (Stein *et al*, 2007). Latha *et al* (2004) reported the development of markers for allele mining of stress tolerance genes in rice germplasm. As the first wheat disease resistance genes have been cloned (Huang *et al*, 2003; Feuillet *et al*, 2003; Yahiaoui *et al*, 2004; Yahiaoui *et al*, 2006; Cloutier *et al*, 2007), the sequence information of these genes should allow the rapid analysis of the genetic diversity at

these loci over a wide range of germplasm and the subsequent identification of new alleles through allele mining.

Pm3 is the only wheat powdery mildew resistance gene cloned to date. This locus occurs in seven different alleles that confer race-specific resistance against *Blumeria graminis* f.sp. *tritici* (*Bgt*). Based on sequence information from the first cloned *Pm3b* allele, the remaining six *Pm3* resistance alleles i.e., *Pm3a*, *Pm3c*, *Pm3d*, *Pm3e*, *Pm3f*, and *Pm3g* (Yahiaoui *et al*, 2004; Srichumpa *et al*, 2005; Yahiaoui *et al*, 2006) were isolated from wheat breeding lines. *Pm3* alleles encode resistance proteins with a nucleotide-binding (NB) and a leucine-rich repeat (LRR) domain associated at the N-terminus with a coiled-coil (CC) domain. The high sequence conservation of the alleles suggested their recent evolution from a common ancestor sequence called *Pm3CS*. *Pm3CS* is found in many wheat lines and represents a susceptible *Pm3* allele (Yahiaoui *et al*, 2006).

We have previously characterised the occurrence and geographical distribution of the known *Pm3* alleles in a subset of 211 powdery mildew resistant wheat landraces from a large collection established following a FIGS strategy (Kaur *et al*, 2008). *Pm3b* was the most frequent allele (found in 30 landraces), followed by *Pm3c* (4 landraces) whereas the other alleles were not found in the subset. In addition, powdery mildew resistant lines were identified which could be used as potential material for isolation of new *Pm3* alleles (Kaur *et al*, 2008).

Here, we describe the successful and efficient screening of wheat gene bank accessions for the molecular identification of allelic variants at the *Pm3* locus of wheat. We report the cloning of seven new functional *Pm3* alleles from a targeted subset of wheat landraces that was established by FIGS. We also demonstrate that at least two of the new *Pm3* alleles isolated from landraces confer slow acting resistance. The strategy we describe here can be implemented for other diversity and molecular breeding studies involving agriculturally important traits.

3. Results

3.1 Screening a focused subset of wheat landraces for novel *Pm3*-based powdery mildew resistance

To maximize the chances of finding variation for powdery mildew resistance while limiting the number of accessions to a workable size, we first defined a subset from large wheat collections in gene banks using the Focused Identification of Germplasm Strategy (FIGS). From a total of 16'089 accessions to choose from, the FIGS process identified a set of 1320 (8.2% of total collection) accessions drawn from 323 sites that included accessions from Turkey (419), Iran (391), Afghanistan (292), Pakistan (133), Armenia (34), Turkmenistan (16), Russia (9), India (6), Azerbaijan (1) and Uzbekistan (1). To select powdery mildew resistant accessions, the whole set of 1320 landraces was screened with four different powdery mildew isolates (Kaur *et al*, 2008). Among the 211 landraces found resistant or intermediately resistant to at least one of the isolates tested, 40% originated in Turkey, 21% in Afghanistan, 14% in Iran and the rest were distributed in the Caucasus, Central Asia and Pakistan. These 211 landraces were further submitted to two successive steps of molecular analysis. First, the screening for the presence of a *Pm3*-like gene with a diagnostic STS marker and secondly, the lines positive for STS marker were screened for the presence of the already known alleles (Kaur *et al*, 2008). This led to identification of 111 landraces positive for the *Pm3* diagnostic fragment but lacking any of the already known *Pm3* alleles. These accessions were the best candidates for the isolation of new *Pm3* alleles although it could not be ruled out that the resistance is caused by other *Pm* genes.

3.2 Cloning of new *Pm3* alleles from selected wheat landraces

The isolation of *Pm3* alleles was performed on 56 landraces which were completely resistant to at least one *Bgt* isolate whereas the lines with intermediate resistance were not considered further. The *Pm3* coding sequences were successfully isolated from 45 landraces and cloned. In

the remaining landraces, amplification of a *Pm3* coding sequence was not possible which might be due to absence of the gene or low sequence homology at the primer binding sites. All the 45 isolated genes were sequenced and compared. The analysis of sequence diversity led to the identification of sixteen new allelic sequences of the *Pm3* powdery mildew resistance gene, as several landraces possessed identical alleles (Figure 5.1 and Appendix 9.1). Fifty percent of the new alleles i.e., eight out of 16 (*Pm3_42416*, *Pm3_42920*, *Pm3_42525*, *Pm3_42277*, *Pm3_42868*, *Pm3_42255*, *Pm3_42281* and *Pm3_42469*) originated from landraces collected in Turkey. The second highest number of new alleles originated from Afghanistan (4 alleles: *Pm3_9939*, *Pm3_10963*, *Pm3_14475*, *Pm3_14442*) while *Pm3_31594*, *Pm3_23728* and *Pm3_41606* originated from Azerbaijan, Turkmenistan and Pakistan, respectively. Different landraces with identical *Pm3* alleles always originated from the same country, with one exception *Pm3_13636* found in two landraces originating from very different locations, i.e., Afghanistan and Turkey. Among the 45 sequences, 9 were identical to the susceptible *Pm3CS* allele (Yahiaoui *et al*, 2006) which was present in accessions from different countries (2 from Pakistan, 2 from Turkey, 1 from Iran and 4 from Afghanistan). This indicates that the resistant phenotype observed in these landraces is not due to a gene at the *Pm3* locus but may be caused by any of the known or still uncharacterized *Pm* genes in the germplasm.

3.3 Sequence diversity at the *Pm3* locus

The DNA sequence of the 16 new alleles was compared to the known *Pm3* alleles (*Pm3a* to *Pm3g* and *Pm3CS*; Figure 5.1). The structural arrangement of the new *Pm3* sequences corresponds well to the one described for the known *Pm3* alleles. The alleles consist of two exons separated by an intron of 200bp and encode for a CC-NBS-LRR type of protein.

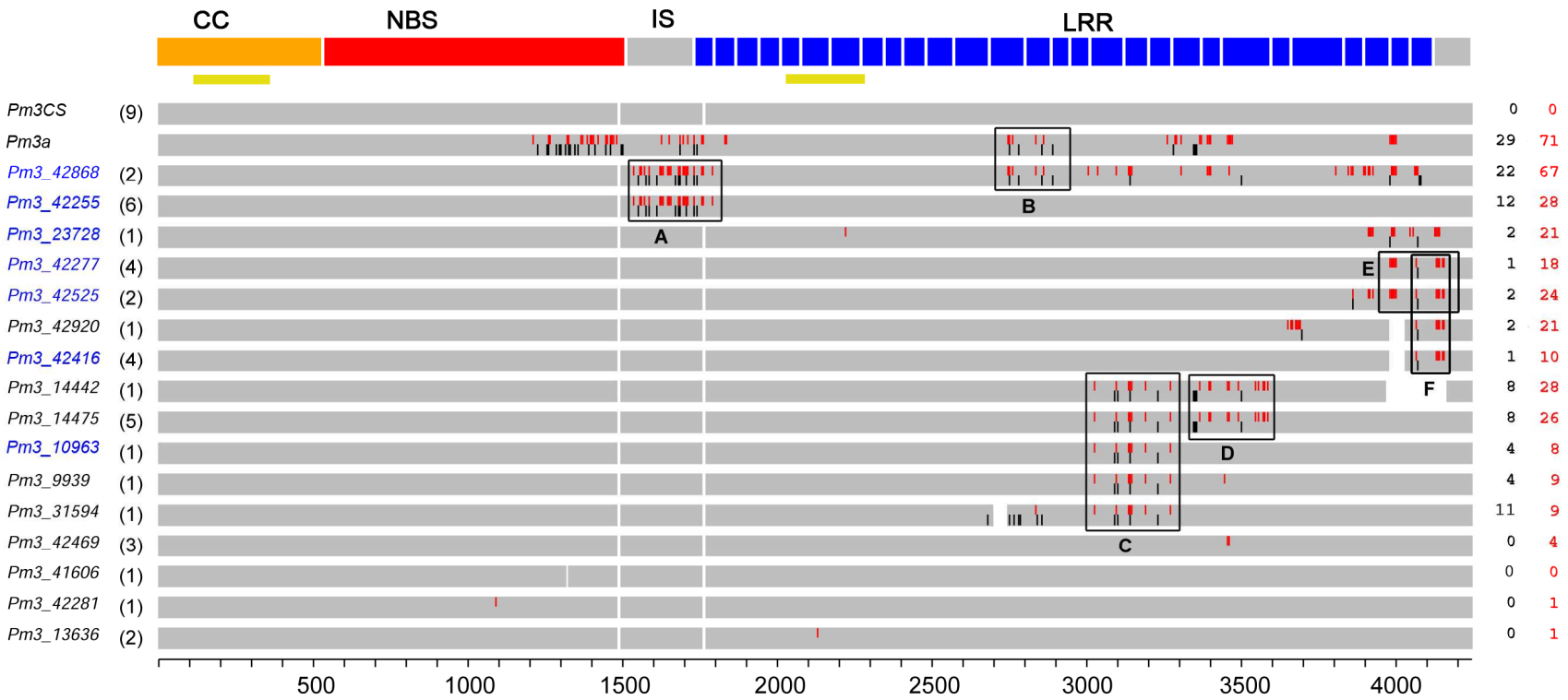

Figure 5.1: Schematic representation of the *Pm3* gene structure (only exons) and sequence alignment of new *Pm3* alleles with the known alleles *Pm3*a and *Pm3CS*.

The domains encoded by the *Pm3* alleles are depicted at the top (CC, NBS, Interspacer, LRR). Red bars in the *Pm3* alleles and numbers in red indicate the polymorphic nucleotides as compared to *Pm3CS* leading to non-synonymous changes in the protein. Black bars and numbers in black represent the polymorphic nucleotides leading to synonymous mutations. The boxes A, B, C, D, E, F indicate the putative gene conversions tracts among these alleles. The numbers in parenthesis correspond to the number of landraces possessing that particular *Pm3* allele. The golden bars indicate the regions for VIGS constructs. The functional alleles, *Pm3_42416*, *Pm3_42525*, *Pm3_23728*, *Pm3_42255*, *Pm3_10963*, *Pm3_42277* and *Pm3_42868* are labeled in blue colour.

Nine of the new alleles are 4442 bp long. This corresponds to the size of the ancestral reference sequence of *Pm3CS*. The remaining seven alleles bear InDels (*Pm3_42868, Pm3_42255, Pm3_42416, Pm3_42920, Pm3_31594, Pm3_41606* and *Pm3_14442*) which make them variant in size. Single three bp indels occur in exon1 of *Pm3_42868* and *Pm3_42255* (full length 4445bp) and of *Pm3_41606* (full length 4439 bp). An identical 45 bp deletion in exon1 is shared by *Pm3_42416* and *Pm3_42920* while exon 1 of *Pm3_31594* bears a 45 bp deletion at a different position. The PM3 proteins usually have 28 LRR repeats each with the conserved LXXLXLXX motif. Here, *Pm3_42416* and *Pm3_42920* miss the sequence encoding for the LRR 27 in other alleles while in *Pm3_31594,* the LRR 13 encoding region is absent. All these InDels did not alter the open reading frames. Only *Pm3_14442* is a pseudogene where 294 bp spanning the end of exon1 and the first part of the predicted intron are deleted leading to a frame shift. The deletions observed in the alleles appear to be the result of illegitimate recombination (IR). We identified a 4 bp imperfect repeat motif at the breakpoint of the deletion in *Pm3_42416* and *Pm3_42920* that could have served as template for the IR. Devos *et al* (2002) reported that mismatches in the direct repeats that serve as templates for IR are found frequently. In *Pm3_ 31594,* a perfect 3 bp repeat exists at the breakpoint of the deletion suggesting illegitimate recombination.

The CC-NBS encoding region of the new alleles is highly conserved with only 3 bp deletion and one single base change in the NBS region of *Pm3_41606* and *Pm3_42281,* respectively (Figure 5.1). Most polymorphic nucleotides between the new alleles and in comparison to *Pm3CS*, lie in the region encoding the LRR domain, with exceptions for *Pm3_42255* and *Pm3_42868* which also show variability in the spacer region between the NBS and LRR region. The average nucleotide diversity (π) for the whole coding sequence among the 16 new *Pm3* sequences was calculated as 0.0078 which is lower than nucleotide diversity reported for the known *Pm3* alleles (π=0.012, Yahiaoui *et al*, 2006).

The intron is identical among the majority of the new alleles and the known *Pm3* alleles except for 5 new alleles where it differed by at the most 6 base positions. Five nucleotide sites were polymorphic for *Pm3_42416*, *Pm3_42277*, *Pm3_42525* and *Pm3_42920*, while for *Pm3_23728*, 2 sites among the 5 mentioned plus one additional (3 total) are different.

The comparison of allelic sequences shows basically two groups of sequences. The first group of 4 sequences (*Pm3_42469*, *Pm3_41606*, *Pm3_42281* and *Pm3_13636*) has few or single polymorphic residues compared to the *Pm3CS* reference sequence. In the second group, polymorphic residues are present as sequence blocks (blocks A to F, Figure 5.1) which are completely or partially shared between alleles. *Pm3_42255* and *Pm3_42868* possess an identical block of sequence with 42 substitutions (block 'A'). Within this stretch the two alleles share identical residue changes at 15 sites with the *Pm3a*, *Pm3b* and *Pm3f* alleles in comparison to *Pm3CS*. *Pm3_42868* also shares a second similarity block with *Pm3a* and *Pm3f* (block 'B'). *Pm3_42868* is the allele with the highest number of identical residue change sites shared with the known *Pm3a*, *Pm3b* or *Pm3f* alleles. Thus, it might have served as an ancestor in the evolution of *Pm3a*, *Pm3b* or *Pm3f*. The 5 alleles *Pm3_14442*, *Pm3_14475*, *Pm3_10963*, *Pm3_9939*, and *Pm3_31594* share a polymorphic sequence block (block 'C') which spans the regions encoding LRR17 till LRR19. This block is followed in *Pm3_14475* and the pseudogene *Pm3_14442* by an identical strech of polymorphic residues (block D). *Pm3_42277* and *Pm3_42525* possess a similar block (block 'E') with 18 non-synonymous residue changes. The last eleven substitutions of block E are present in *Pm3_42416* and *Pm3_42920* and form block 'F' while at three of these six sites *Pm3_23728* possesses a different residue. This indicates the frequent occurrence of sequence exchange between alleles possibly by gene conversion. The number of non-synonymous mutations is higher than the synonymous mutations among the polymorphic

sequence blocks. This is also true in general for the complete coding sequence of the new alleles except for *Pm3_31594*.

The differences in proteins encoded by the newly isolated *Pm3* alleles, lead to a total of 87 amino acid changes compared to *PM3CS*, in addition to the InDels. At a majority of these sites in the new alleles, single residue changes (72 of 87 positions) were shared by different alleles, with few sites having two or three alternative residues, i.e., 13 and 1 (R/D/Y at position 1332 instead of W in PM3CS) out of 87, respectively. In the complete LRR domain, 27 among the 59 residue changes (45.7%) lie in the solvent exposed residues (X in LXXLXLXX).

3.4 Functional analysis of *Pm3* candidate resistance genes by Virus Induced Gene Silencing

As the resistance in the lines containing newly isolated *Pm3* alleles might be caused by *R* genes other than *Pm3*, we used virus induced gene silencing (VIGS) to determine if the resistance is based specifically on the new *Pm3* alleles or if other *Pm* genes are additionally present. The infection of wheat with the Barley Stripe Mosaic Virus (BSMV) containing a gene fragment results in silencing of the corresponding endogenous gene (Holzberg *et al*, 2002; Scofield *et al*, 2005). We designed three VIGS constructs that contain target gene fragments of about 300 bp in length. Two constructs targeting *Pm3* alleles contained fragments of the CC (BSMV.*Pm3*_CC) and of the LRR domain (BSMV.*Pm3*_LRR) respectively (golden bars mark these regions in Figure 5.1). A control construct carried a fragment of CC region of the *Lr10* leaf rust resistance gene. Six landraces carrying new *Pm3* allelic sequences (*Pm3_42416*, *Pm3_10963*, *Pm3_42525*, *Pm3_23728*, *Pm3_42469* and *Pm3_42255*) were infected with the constructs BSMV.Lr10_CC (control), BSMV.*Pm3*_CC and BSMV.*Pm3*_LRR and were further challenged with avirulent *Bgt*

isolate 98275. A set of plants challenged with *Bgt* isolate 98275 but without BSMV infection served as additional control.

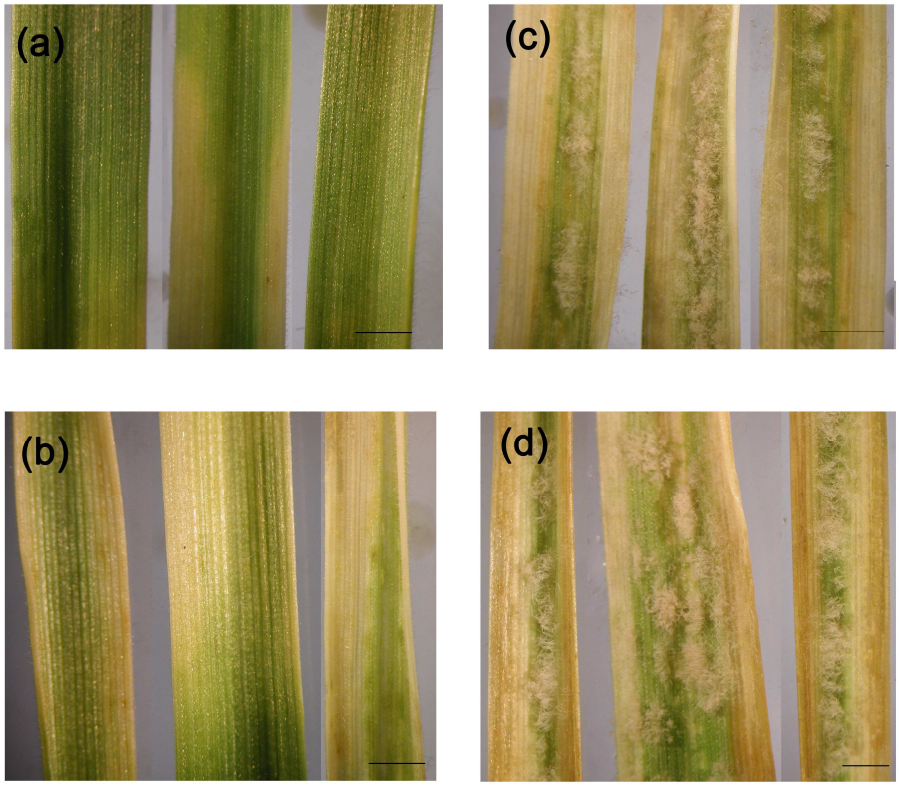

Figure 5.2: BSMV-mediated VIGS of *Pm3_42416* and *Pm3_42255*.
(a) The landrace containing *Pm3_42416* infected with powdery mildew but not with the virus serves as resistant control. (b) The infection with BSMV.*Lr10*_CC (control viral construct) does not alter the observed resistance against mildew. (c,d) The resistant landrace turns susceptible to powdery mildew when infected with BSMV.*Pm3*_CC and BSMV.*Pm3*_LRR, respectively. Similar observations were made for *Pm3_42255*. This shows the silencing of target *Pm3* alleles in these landraces. Bars correspond to 0.5 cm.

The landraces carrying the alleles *Pm3_42416* and *Pm3_42255* (IG42416 and IG42255 respectively) lost resistance to *Bgt* isolate 98275 after infection with BSMV.*Pm3*_CC and

BSMV.*Pm3*_LRR (Figure 5.2). These two landraces remained resistant when inoculated with the control BSMV.*Lr10*_CC. This demonstrated that the *Pm3_42416* and *Pm3_42255* genes in these lines were conferring resistance against *Bgt* isolate 98275. In contrast, resistance was not altered in the landraces with the alleles *Pm3_42469*, *Pm3_42525* and *Pm3_10963* indicating that in these landraces powdery mildew resistance might also be mediated by additional genes other than *Pm3*. In the case of allele *Pm3_23728*, results were not conclusive possibly due to a heterogenous seed mixture for this accession.

3.5 Identification of functional *Pm3* alleles by transient transformation

In addition to VIGS analysis, we tested the new *Pm3* alleles in a transient transformation assay in order to determine the contribution of these alleles towards the observed resistance. This assay is particularly important in case of alleles for which VIGS was not conclusive or resistance was not altered. The transient gene expression assay (Schweizer *et al*, 1999; Yahiaoui *et al* 2004) is based on ballistic transformation of single leaf epidermal cells, followed by subsequent inoculation of transformed leaves with specific powdery mildew isolates. Using this system, we have tested 13 alleles from landraces for function. The coding sequences of the *Pm3* alleles were co-bombarded with a plasmid carrying the β-glucuronidase (GUS) reporter gene into leaf epidermal cells of the powdery mildew-susceptible wheat line Chancellor. For selection of the powdery mildew isolate for the transient assay, we took advantage of the fact that the same allele was identified in several landraces. Therefore, the isolate to which all independent landraces with identical alleles show resistance was used to infect the bombarded leaves. Eight alleles i.e., *Pm3_42416*, *Pm3_42920*, *Pm3_9939*, *Pm3_10963*, *Pm3_42525*, *Pm3_23728*, *Pm3_42255* and *Pm3_42469* were tested with isolate 98275, four alleles i.e., *Pm3_31594*, *Pm3_42277*, *Pm3_42868*, *Pm3_14442* with *Bgt* 97011 and one allele, *Pm3_41606*, with *Bgt* 96224. It was not possible to identify an appropriate isolate for *Pm3_14475*, *Pm3_13636* and *Pm3_42281* because independent landraces with these

alleles behaved differently to the tested isolates suggesting that the observed resistance was not due to the *Pm3* allele. As a control for the experiments, we used the non-functional *Pm3CS* allele (Yahiaoui *et al* 2006). The percentage of compatible (susceptible) interactions, i.e. the haustorium index, was determined at 48hpi. In the control experiments, a high percentage (70 to 76 %) of the cells expressing the GUS reporter gene and attacked by one powdery mildew spore showed a fully developed haustorium indicative of a compatible interaction. In contrast, seven alleles *Pm3_42416*, *Pm3_42525*, *Pm3_23728*, *Pm3_42255*, *Pm3_10963*, *Pm3_42277* and *Pm3_42868* showed a significant reduction in haustorium index compared to the susceptible control *Pm3CS* (Figure 5.3a).

Two of them, *Pm3_42416* and *Pm3_42255* showed the most significant reduction in the haustorium index (42.7% and 42.6%, respectively in comparison to corresponding *Pm3CS* values of 74.9% and 75%). Transformation with the remaining 6 out of 13 alleles tested (*Pm3_42920*, *Pm3_9939*, *Pm3_42469*, *Pm3_31594*, *Pm3_14442*, *Pm3_41606*) did not result in a reduction of the haustorium index (data not shown). To check for race specificity of the resistance conferred by the new alleles, the two strongest alleles showing most significant reduction in haustorium index in the transient system (*Pm3_42416* and *Pm3_42255*) were also tested against a virulent isolate 97019. No reduction of haustorium index was observed compared to *Pm3CS* (Figure 5.3b) demonstrating that the observed activity was not due to over expression but to race specific resistance conferred by these genes.

In conclusion, the alleles *Pm3_42416*, *Pm3_42255*, *Pm3_23728*, *Pm3_10963*, *Pm3_42525*, *Pm3_42277* and *Pm3_42868* are new, functionally active forms of *Pm3* which are called *Pm3l*, *Pm3m*, *Pm3n*, *Pm3o*, *Pm3p*, *Pm3q* and *Pm3r* respectively, extending the *Pm3* allelic series of

resistance genes. Five out of these seven alleles originated from Turkey, whereas *Pm3*_10963 and *Pm3*_23728 are from Afghanistan and Turkmenistan, respectively.

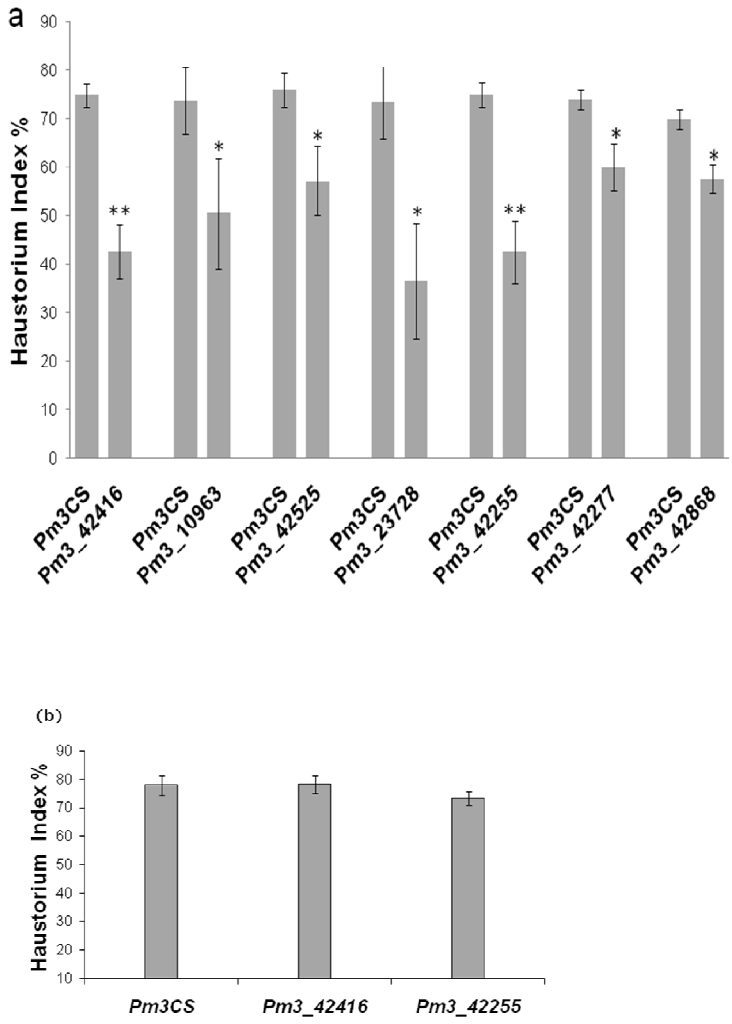

Figure 5.3: Results of the functional analysis of the new *Pm3* alleles in the transient transformation assay.

Genomic sequences of the new *Pm3* alleles or control genes were co-bombarded with the GUS reporter gene. The haustorium index (percentage of cells with haustoria) is indicated by the mean ± SD of three independent experiments, each contributing at least 50 interactions. * Significant differences at p= 0.05; ** significant differences at p=0.001, are indicated for the tested *Pm3* alleles in comparison to the susceptible control *Pm3CS*. (a) Transient assay results for the seven new *Pm3* alleles tested with corresponding avirulent *Bgt* isolates. (b) Transient assay result for *Pm3_42416* and *Pm3_42255*, upon infection with the virulent isolate 97019. No significant difference was observed.

3.6 Are the new *Pm3* alleles late acting resistance genes?

In comparison to the known *Pm3* alleles (Yahiaoui *et al*, 2004, Srichumpa *et al*, 2005), the newly identified alleles have relatively high haustorium indices. To determine if this resistance reaction in the transient assay is due to slow action of the new alleles, we performed LPTB (Lacto-phenol trypan blue) staining in two landraces with new *Pm3* alleles. We monitored pathogen growth and HR at six different time points between two and seven days post inoculation. The staining was performed on wheat landraces carrying the new *Pm3_42416*/*Pm3l* and *Pm3_42255*/*Pm3m* alleles as these showed most significant reduction in the haustorium index when tested in the transient assay and were also silenced with VIGS, confirming that the observed resistance in these landraces is caused specifically by *Pm3* alleles. The primary leaves of these landraces were challenged with avirulent *Bgt* isolate 98275 and later stained at the different time points. Cultivar Chul carrying *Pm3b* was included as a comparison to a known allele in elite material.

Figure 5.4 (a & b) show the percentage of cells with haustorial formation versus the percentage of haustoria bearing cells showing hypersensitive cell death, at 2, 3, 4, 5, 6 and 7 dpi. Successful formation of secondary hyphae and haustoria in the attacked host epidermal cells was observed at a frequency of 46% and 30% at 2dpi (48hpi) for *Pm3_42416* and *Pm3_42255*, respectively. This time point (48hpi) corresponds to the one in the transient assay where the transformed leaves are arrested for staining. Therefore, the relatively low rate of resistance found in the transient assay for the *Pm3* alleles in these lines is in agreement with the degree of resistance observed *in planta*. At later infection time points, for *Pm3_42416* almost all cells having a haustorium were associated with hypersensitive cell death at 5dpi while for *Pm3_42255* the same was true at 3dpi (Figure 5.4). An overall reduction in the number of cells with haustorium was observed in case of *Pm3_42255* at 7dpi, which may be attributed to death of fungus by this time. In the case of Chul, no compatible interactions were observed at any time point indicating a strong resistance-

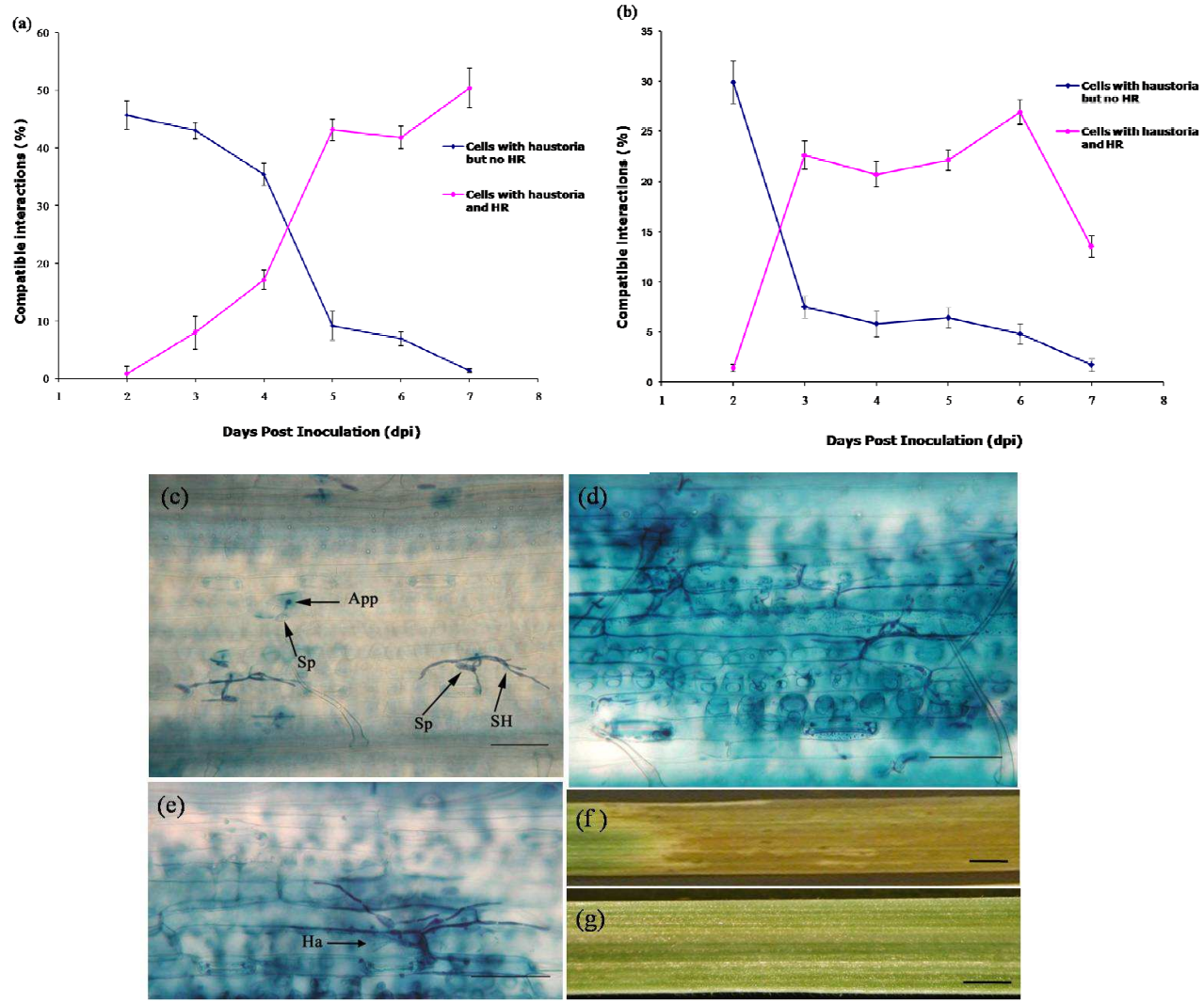

Figure 5.4: Time course analysis of pathogen growth on the landraces with *Pm3_42416* or *Pm3_42255*.

Trypan blue staining was done on the leaves of landraces with alleles *Pm3_42416* and *Pm3_42255*. The graphs (a, b) show the percentage of cells with haustoria in the epidermal cells of landraces with *Pm3_42416* (a) and *Pm3_42255* (b), respectively. The blue line indicates the haustorium index calculated from the cells with haustorium showing no HR, while the pink line marks the cells with haustoria that also exhibit HR. These observations were recorded at different time points (2, 3, 4, 5, 6 and 7 dpi) indicated at the bottom of the graph. (c) Microscopic view of cells from landrace with *Pm3_42416* at 2dpi. This shows the occurrence of interactions with and without haustoria formation while none of these leads to cell death at this stage. (d,e) The cells with haustoria are accompanied by HR at 5dpi. A blue stained cell indicates cell death resulting from HR. Macroscopic view of the infected leaf segments from landraces with *Pm3_42416* and *Pm3_42255* at 8dpi (f,g), respectively. Arrows indicate, Sp: spore, SH: secondary hypae, App: appresorium, Ha: haustorium. Bars in 5.4c-e correspond to 100µm and in 5.4f-g correspond to 0.5cm.

- response of *Pm3b* already at 48hpi. These results show that *Pm3_42416* is highly active 5 days post inoculation while *Pm3_42255* comes to action 3 days post inoculation. The resistance triggered by these genes is mainly based on a late hypersensitive cell death response that occurs after fungal penetration and development of secondary hyphae as well as extensive haustoria formation. As a consequence of this, the landraces with *Pm3_42416* and *Pm3_42255* were macroscopically clearly resistant with no sign of fungal growth at 8 days post inoculation (Figure 5.4). *Pm3_42416* showed complete death of the leaf resulting from HR while *Pm3_42255* appeared normal green with no visual chlorotic areas, possibly because of earlier arrest of fungal growth.

3.7 Comparison of expression levels: The new *Pm3* alleles versus known *Pm3* alleles

As shown above, the intermediate haustorium indices of the new alleles observed in the transient assay compared to the known *Pm3* alleles are very likely due to slow response of these alleles in pathogen arrest. A possible reason for this delay in the full response could be low expression levels of these genes. Therefore, Semi-quantitative RT-PCR was carried out to compare levels of differentially expressed mRNAs in the landraces possessing the two new active alleles (*Pm3_42416*/*Pm3l* and *Pm3_42255*/*Pm3m*) and the known *Pm3* alleles (*Pm3b, Pm3e, Pm3g, Pm3CS*). The line Chancellor served as a control with no *Pm3* gene. The *Pm3* primers used in the analysis were located one on each of the two exons of *Pm3*. They amplified a 820bp genomic fragment (including the 200bp intron) and a 620bp fragment from cDNA. The expected fragment length was 45 bp smaller in *Pm3_42416* because of the deletion in this allele. As shown in figure 5.5, no difference in the expression levels of the *Pm3* alleles was observed, revealing that the new alleles are expressed at very similar levels as the already characterized *Pm3* alleles.

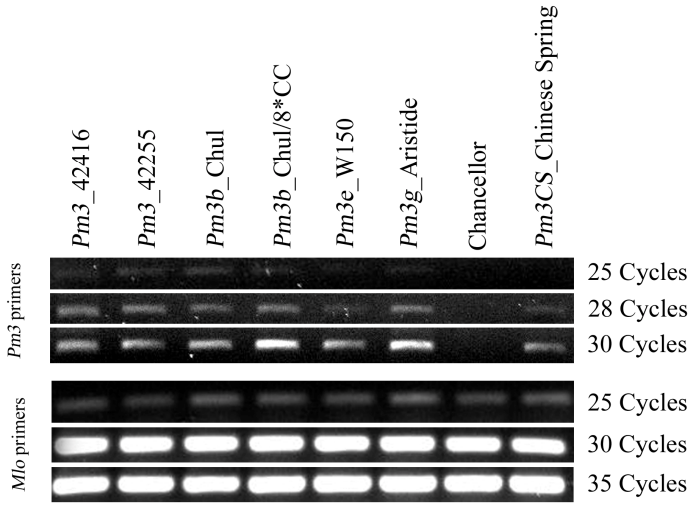

Figure 5.5: Semi-quantitative PCR analysis of *Pm3* expression in landraces with new *Pm3* alleles in comparison to lines having known *Pm3* alleles.

Semi-quantitative PCR of *Pm3_42416* and *Pm3_42255* was performed in comparison to lines with *Pm3b, Pm3e, Pm3g* and *Pm3CS*, by using *Pm3* specific primers. The line Chancellor served as negative control with no *Pm3* gene. The amplification with 3 different number of cycles indicate very similar levels of expression for the new as well as known alleles. The *Mlo* primers were used as control for quality and quantity of cDNA used for the PCR.

3.8 The new alleles have different specificities as compared to known *Pm3* alleles

To determine whether the two new *Pm3* alleles show novel specificities if compared to already known *Pm3* alleles, we subjected the landraces carrying *Pm3_42416* and *Pm3_42255* to further characterization by infection with a set of six additional powdery mildew isolates, resulting in a total of 10 isolates tested. A differential response to powdery mildew isolates was observed for these landraces compared to known *Pm3* alleles. A resistance phenotype to *Bgt* 96229 was observed with lines containing *Pm3_42416, Pm3_42255, Pm3a, Pm3b*, *Pm3c*, *Pm3e* and *Pm3f* while the lines carrying *Pm3d* and *Pm3g* were susceptible to this isolate. In contrast, the lines with *Pm3_42416* and *Pm3_42255, Pm3d* and *Pm3g* were susceptible to *Bgt* 97019 while lines with all the other alleles were resistant to this isolate. Other isolates were identified that differentiate the new alleles from the known alleles (See appendix 9.2). In addition, one *Bgt* isolate '95.45 Brimstone' differentiated the two new alleles *Pm3_42416* and *Pm3_42255* from

each other: *Pm3_42416* was resistant while *Pm3_42255* was intermediately susceptible. Based on the these data, we conclude that these two newly isolated alleles encode new *Pm3* specificities and could be potentially useful in wheat breeding, broadening the spectrum of possible resistance combinations.

3.9 Domain swapping to understand the role of a small deletion in resistance

Pm3_42416 differs from *Pm3CS* only with a deletion of 45 bp and a stretch of 15 nucleotide substitutions in exon1 and the intron (Figure 5.6a). As described above, illegitimate recombination has possibly been the cause of this deletion in the LRR domain (Figure 5.6b). In order to determine whether the 45bp deletion or the polymorphic residues in *Pm3_42416* (or both) have a role in the resistance function, we performed domain swaps between the *Pm3_42416* and *Pm3CS* genes. The deletion of *Pm3_42416* corresponds to the region encoding LRR27 while the non-synonymous substitutions are present in region encoding LRR28 and close to the end of the gene (leading to 1 and 5 amino acid changes, respectively). Two domain swap constructs were made, wherein swap1 was *Pm3CS* with the *Pm3_42416* deletion of 45 bp, while the second construct (swap2) was *Pm3CS* with the 15 base pair changes of *Pm3_42416* (Figure 5.6a).

Transient expression analysis was carried out for these two constructs with the original parental genes *Pm3CS* and *Pm3_42416* used as controls. The results showed that both the domain swaps impart resistance against *Bgt* isolate 98275 (Figure 5.6c). Haustorium index values were of 74.2% for the *Pm3CS* susceptible control, versus 48% for *Pm3_42416*. For the swap1 construct, an intermediate reduction in haustorium index was observed (62.2%). The swap2 construct resulted in resistance which was in the same range as the parental gene *Pm3_42416* (49.36% of haustorium index), indicating that the 15 base pair substitutions are sufficient to provide this resistance. Nevertheless, the 45 bp deletion by itself also seems to confer some resistance activity.

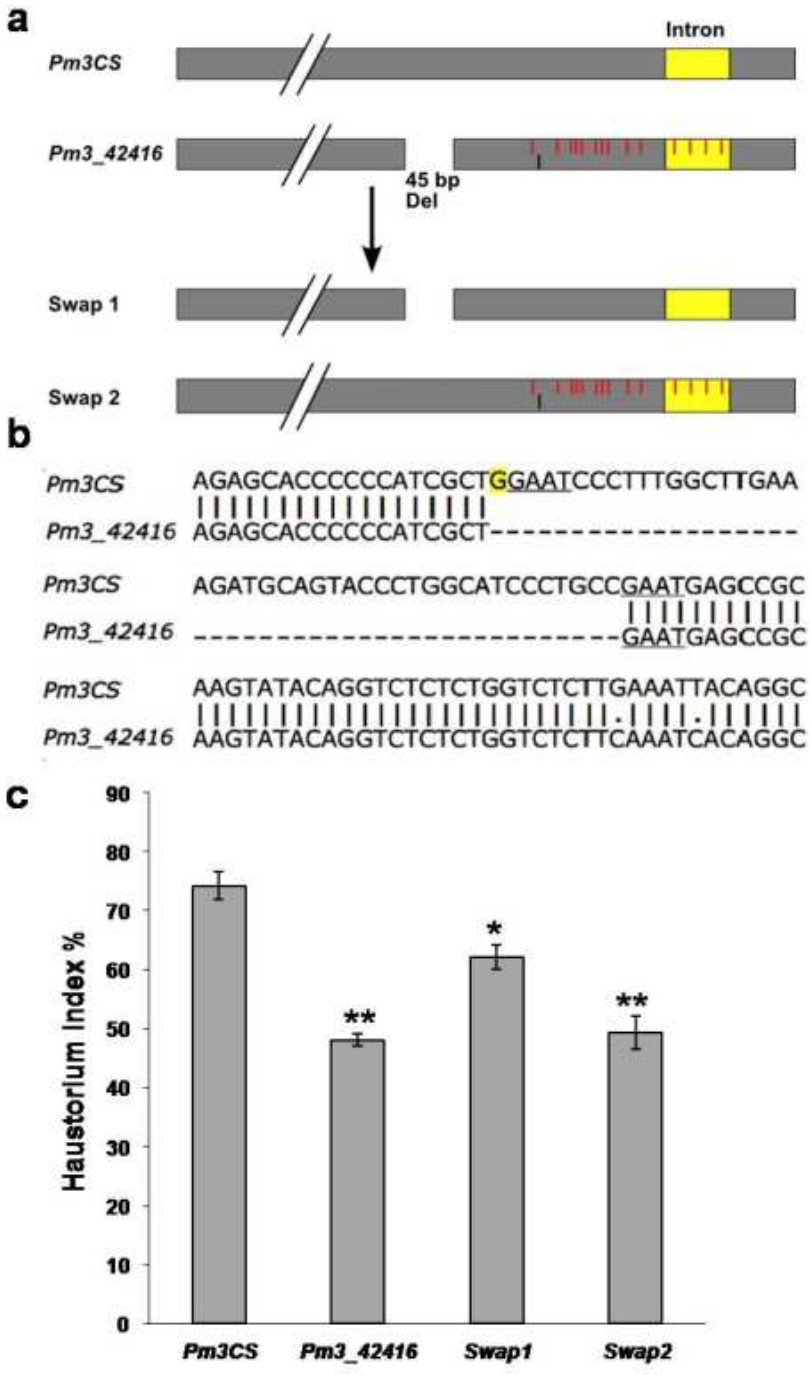

Figure 5.6: Domain swap experiment for the analysis of the role of polymorphic regions between the *Pm3_42416* and *Pm3CS* alleles.

(a) Schematic representation of the chimeric constructs made from *Pm3_42416* and *Pm3CS*. Swap1 contains the deletion of *Pm3_42416* in the background sequence of *Pm3CS* while Swap2 has the polymorphic nucleotides of *Pm3_42416* in the *Pm3CS* background. (b) The nucleotide sequence alignment indicating that illegitimate recombination caused the deletion in *Pm3_42416*. *Pm3CS* is used as the template sequence. A 4 bp motif that could have served as a template for IR is indicated. (c) Transient assay results of the two domain swap constructs in comparison to the two parental sequences *Pm3_42416* and *Pm3CS*. The function of the gene constructs in the transient assay is evaluated by the haustorium index (% of compatible interactions).* Significant differences at p= 0.05; ** significant differences at p=0.001, are indicated for the tested *Pm3* constructs in comparison to the susceptible control *Pm3CS*.

4. Discussion

4.1 *Pm3* allele mining in a subset of landraces specifically selected for the isolation of powdery mildew resistance genes

With more than 560,000 wheat accessions held in nearly 40 genebanks globally (The Global Crop Diversity trust, 2007; http://www.croptrust.org/documents/web/Wheat-Strategy-FINAL-20Sep07.pdf), it is not a realistic scenario to screen the entire collection for specific traits or genes. The question then becomes what is the most effective way to choose a set of material to screen. The selected set has to be of a manageable size that will have a reasonable probability for containing the relevant trait, for example disease resistance. The definition of a core collection is frequently proposed as a strategy to work with fewer accessions that would represent the genetic diversity of a crop species and its relatives in the entire collection with a minimal repetitiveness (Ortiz, 2002; Warburton *et al*, 2008). However, as Mackay (1995) argued, while core collections aim to maximize the genetic diversity (of some larger collection) in a smaller number of accessions, breeders and pre-breeders usually only seek one or a few traits at a time when approaching a genetic resource collection. Indeed, this was the case in the present study as we were only seeking variation for powdery mildew resistance. Mackay and Street (2004) described the focused identification of germplasm strategy that utilizes information about the environment from which wild and landraces accessions were collected to predict where selection pressures for adaptive traits may occur. This information is then used to target sections of genetic resource collections to create trait specific best-bet sets.

The rationale behind FIGS is based on the fact that the environment strongly influences gene flow, natural selection and thus spatial/geographic differentiation (Spieth 1979; Epperson 1990). In the case of powdery mildew, Paillard *et al* (2000) reported that those populations of winter

wheat with the highest level of resistance to powdery mildew originated from sites where powdery mildew pressure was high, due to environmental factors, while the reverse was true of those populations where the pressure was low. Advantageous gene variants might therefore be found in wild relatives and landraces from stressful habitats (Takeda and Matsuoka, 2008).

Landraces are considered to be in the primary pool of genetic resources for wheat. In our study, we have performed a large scale allele mining for powdery mildew resistance on a set of landraces which were selected through FIGS. Indeed, this study demonstrated the effectiveness of the FIGS approach to identify a manageable sub-set of material for screening. Forty percent of the collection sites chosen in the process yielded accessions that were resistant. Of the accessions chosen, almost 16% carried resistance with a surprising high frequency of variation in the *Pm3* loci. Again this is an excellent result from a subset of less than 8% of the total pool of accessions chosen for the study. In the search for more variation in the powdery mildew resistance loci we propose that the results of this study are used to further refine the FIGS selection process.

Allele mining or EcoTILLING is being applied in plant or crop species as diverse as melon (Nieto *et al*, 2007) and poplar (Gilchrist *et al*, 2006). There are ongoing allele mining projects,e.g in barley (Stein *et al*, 2007) about 2000 accessions are being screened to identify allelic variants of translation initiation factor *Hv-eIF4E*, which confer resistance to Bymoviruses.

We followed a hierarchical selection procedure combining a phenotypic screen and molecular markers specific for the *Pm3* haplotype, to select for the resistant lines within the FIGS set which are likely to possess *Pm3* alleles and we ruled out the presence of already known *Pm3* alleles in these candidate lines by using the allele specific primers (Kaur *et al*, 2008). We have identified 16 new *Pm3* sequences from 45 resistant landraces among which 43% were found functionally active. These seven functionally active *Pm3* alleles, *Pm3l, Pm3m, Pm3n, Pm3o, Pm3p, Pm3q* and *Pm3r* extend the previously known *Pm3* allelic series. Five of seven new alleles found functional

originated from Turkey while the remaining two alleles originated from Afghanistan and Turkmenistan. The classical *Pm3* alleles (*Pm3a* to *Pm3g*) were identified from different regions in the world, more particularly from Asia (Uzbekistan for *Pm3b*, the Hindukush region for *Pm3d*, Japan for *Pm3a*). The FIGS strategy and our work here identify Turkey as a region that bears the potential to yield functional specificities which were not carried forward into the breeding material in the past.

4.2 A combined strategy for the analysis of the function and specificity of the new *Pm3* alleles

We have used virus induced gene silencing (VIGS) using barley stripe mosaic virus as well as a transient transformation assay for the analysis of the function of new *Pm3* alleles. Though VIGS has proven to be effective method for some dicots (Burch-Smith *et al*, 2004), it is very recent that its successful use in monocots such as barley and wheat has been demonstrated. VIGS was shown to silence barley phytoene desaturase gene (Holzberg *et al*, 2002) and later was successful in silencing of leaf rust genes *Lr21* (Scofield *et al*, 2005) and *Lr1* (Cloutier *et al*, 2007). In our study, we found that VIGS was an effective strategy to assign function to the new alleles. However, VIGS will only give conclusive results if the resistance in a line of interest is based specifically on the silenced gene. Based on our data, a majority of lines resistant to powdery mildew have either more than one resistance gene or resistance was not due to the *Pm3* allele. In these frequent cases, transient or stable transformation must be used for functional analysis of a candidate gene. Nevertheless, if a gene can be silenced by VIGS, the resistance in the donor landrace can immediately be attributed to the gene, allowing further detailed study on resistance activity and specificity as was the case for two of our new *Pm3* alleles.

To detect the function of individual powdery mildew resistance genes in wheat, the transient transformation assay has been demonstrated as an effective method (Schweizer *et al*, 1999;

Yahiaoui *et al*, 2004; Srichumpa *et al*, 2005). When tested through this method, seven new *Pm3* alleles showed significant reduction in haustorium index as compared to the susceptible control *Pm3CS*. However, for all of them, the haustorium indices were intermediate. The LPTB staining demonstrated that resistance functions of two of these alleles are in fact occurring later than 48hrs when staining and observation is made in the transient assay. This resistance occurs by late cell death after secondary hyphae formation. A late hypersensitive cell death associated resistance has also been reported for some barley powdery mildew resistance genes, for example *Mla3* and *Mla7* (Boyd *et al*, 1995; Shen *et al*, 2003) and might be a widespread phenomenon in allelic series of *R* genes. We conclude that at least two of the new alleles isolated from landraces are slow acting alleles while the known *Pm3* alleles provide a rapid resistance response without formation of haustoria (Yahiaoui *et al*, 2004; this study). This delayed resistance does not seem to be due to a difference of expression between *Pm3* alleles. This suggests that slow resistance is a general phenomenon of *Pm3* genes in landraces and might be the reason why these genes were not selected for breeding. In case of *Pm3_42416*, the resistance phenotype resulted in visible extensive damage of the tested leaf and it is possible that this was undesirable for early breeders and thus, counter selected. Infection experiments carried out with ten different isolates, led us to conclude that 2 new *Pm3* alleles (*Pm3_42416* and *Pm3_42255*) possess a novel and possibly different specificity. These two alleles differ from each other and compared to *Pm3CS* and known *Pm3* alleles by one sequence block in the spacer and LRR1 region and by the 45 bp deletion and block F in the extreme C-terminal part of the LRR. Both these regions seem therefore important for function and specificity of the two new alleles *Pm3_42416* and *Pm3_42255*.

4.3 Sequence diversity in the LRR region of *Pm3* alleles underlies the resistance function

The sequence analysis of the newly isolated alleles revealed an identical gene structure and a high level of sequence identity over the entire length of alleles. It was this conservation of *Pm3* gene

structure that allowed the isolation of 16 new *Pm3* alleles, indicating that no large re-arrangements have affected the *Pm3* locus. The 100% conservation of the N-terminal region that encodes for a coiled coil domain among the new as well as the already reported *Pm3* alleles, suggests a highly conserved function of this domain in *Pm3* resistance. The differences within the new alleles and in comparison to *Pm3CS* or known *Pm3* alleles were mainly found in the LRR domain, with an exception of two alleles which have polymorphisms also in the spacer between the NBS and LRR domain. The alleles have a mosaic pattern of sequence blocks which probably are the result of re-arrangement of variation present in the ancestral alleles. Some alleles have defined blocks enriched with polymorphisms in the LRR region that might have arisen from gene conversion or recombination events. The new *Pm3* alleles also show differences in their coding regions which originated due to InDels and point mutations. InDELs were as well found in the functional flax *L* alleles (Ellis *et al*, 1999) and among the various members of the *Mla* family (Wei *et al*, 2002). Based on the identification of conserved small repeats flanking the InDels in *Pm3* alleles, we propose that these InDels might have originated as a result of illegitimate recombination. Illegitimate recombination has been proposed to be new major evolutionary mechanism that is at the basis of the size variability of the LRR domain of *R* proteins (Wicker *et al*, 2007).

The existence of variability mainly in the LRR domain is in agreement with the suggested role of this domain in recognition specificity (DeYoung and Innes, 2006). In the case of flax rust resistance locus *L*, the major sequence and structural variation among the *L* alleles was also found in the LRR domain (Ellis *et al*, 1999). We demonstrated here that polymorphisms in the LRR region were responsible for the function of *Pm3_42416*. Polymorphic residues at the extreme C-terminal part of the LRR and the deletion of 45 bp were each sufficient to confer a resistance

function. This confirms previous work on known *Pm3* alleles which indicated that the C-terminal part of the LRR domain is important for *Pm3* function and specificity (Yahiaoui *et al*, 2006).

4.4 Application of allele mining in FIGS sets for breeding and basic research

It is important to identify the genetic diversity present in the crop gene pools which in turn can provide functional variants of genes. Based on the data presented here, we suggest that FIGS is an effective sampling strategy that could be applied to other disease resistance screening studies as well to more efficiently mine gene bank collections. Allele mining offers a relatively easy and appropriate alternative to identify functional alleles as compared to traditional ways of identifying new sources of resistance. However, the non-availability of the molecular sequence of the majority of agronomically important genes is still a limiting factor. In this exploratory project, we have identified new functional alleles at the *Pm3* locus. As evolution of resistance is not thought to be a single step process, the non-functional *Pm3* alleles isolated might also have served as 'bridge' sequences which were then further modified by gene conversion, recombination or point mutations to produce the functional resistance specificities. The identification of new alleles through allele mining provides highly valuable alleles for future studies on the molecular function and specificity of the *Pm3* allelic series. In addition, the newly identified functional specificities may be combined together or with already existing *Pm3* alleles (pyramiding) to obtain more durable resistance. Thus, these new alleles will enrich the genetic basis for powdery mildew resistance in wheat. On the basis of results of this pilot project, we conclude that FIGS combined with an allele mining approach bears a high potential to be applied to other important crop plants for targeting important traits or genes.

5. Material and methods

5.1 Plant material

A FIGS approach was used to assemble a subset of 1320 bread wheat landraces (FIGS powdery mildew set), from a total of 16,089 accessions present in three different gene banks of the Australian Winter Cereal Collection (AWCC), the International Center for Agriculture Research in the Dry Areas (ICARDA) and the N.I. Vavilov Institute of Plant Industry, Russia (VIR), to specifically capture variation for powdery mildew resistance.

A virtual collection was created by collating the passport data of bread wheat landraces from the three gene banks into a single database. To link the accessions in the virtual collection with the environment from which they were collected, the geographic coordinates were either captured or derived for all collection sites.The eco-geographic profile of 400 accessions with known powdery mildew resistance was used as a template to identify environmentally similar collection sites from the FIGS database of 16'089 landraces (Kaur *et al*, 2008; Mackay *et al*, manuscript in preparation). Individual accessions were selected using multivariate statistical procedures that determined how eco-geographically similar the collection site of a given accession was to the resistant set template.

5.2 Powdery mildew infections and isolates

In order to screen for resistant landraces, detached leaf segments from seven day old plants were placed on phytagar media and subjected to infection with powdery mildew isolates. The scoring was done 9-10 days after infection. The phenotypes were classified into three categories: resistant (R), intermediate [(I) with two further categories: Intermediate resistant (IR) and Intermediate

susceptible (IS)] and susceptible lines (S). The four isolates used for screening 96224, 98275 and 96244, and 2000.15.Syros, were avirulent or virulent on almost all the known *Pm3* alleles, respectively. The detailed phenotypic reaction of lines carrying the known *Pm3* alleles to the 10 isolates used for specificity characterization of new alleles is given in appendix 9.2.

5.3 Isolation of *Pm3* alleles

Alleles were amplified by using *Pm3*-locus specific, long range PCR amplification followed by a nested long range PCR. PCR primers (UP6: 5'-GGCACAGACAAAGCTCTG-3'; N3SP3R: 5'-ACAATCAGGGATCAGGCC-3'; BamH1: 5'-TTAATTGGATCCCAATGGCAGAGCGGGTGGTC-3' and Sal1: 5'-TATATAGTCGACGCTTCAGCTCCGGCAGGCCTG-3') were based on the upstream and downstream sequence of the coding region of the *Pm3b* allele (Yahiaoui *et al*, 2004). PCR amplification of the *Pm3* alleles was carried out with the *Pfu*Ultra high-fidelity DNA polymerase (Stratagene) as described in Srichumpa *et al*, 2005. For the newly identified *Pm3* sequences, two independent PCR reactions were carried out. Amplified fragments were cloned into the multiple cloning site of expression vector PGY1 (Schweizer *et al*, 1999) between a 540bp fragment of the 35SCaMV promoter and the 35SCaMV terminator. DNA sequencing was performed with an Applied Biosystems Capillary Sequencer model 3730.

5.4 Sequence analysis

Sequence assembly was performed using the Gap4 program of the Staden Package (http://staden.sourceforge.net/). The ClustalX software (Thompson *et al*, 1997) was used for sequence alignments which were further analysed in the program Genedoc (http://www.nrbsc.org/gfx/genedoc/index.html). The different R protein domains (CC, NBS, LRR, structural LRR residues and solvent exposed LRR residues in the XXLXLXX motif) were chosen according to Meyers *et al* (2003) and Yahiaoui *et al* (2004). The average nucleotide

diversity (π) among the 16 new *Pm3* alleles was calculated as in Tajima (1989) using program DNaSP (Rozas *et al*, 2003).

5.5 Virus Induced Gene Silencing

PCR amplified fragments from the *Pm3* gene flanked by restriction sites were inserted into the γ-sub-fragment of the viral genome thus allowing the cloning of the fragments in the antisense orientation (Holzberg *et al*, 2002; Scofield *et al*, 2005). The fragments used were identical among the known and new *Pm3* allelic sequences. The infectious constructs of BSMV RNAs were prepared by *in vitro* transcription using T7 DNA-dependent RNA polymerase (mMESSAGE mMACHINE® T7 Kit, Ambion, USA). Transcripts of each of the BSMV genomes were mixed in a 1 : 1 : 1 ratio, so that each inoculation included all the three viral RNA transcripts consisting of RNA α, RNA β and one of the following γ RNAs: γ-Lr10_CC, γ-*Pm3*_CC and γ-*Pm3*_LRR. Wheat seedlings were grown in a climate chamber for 1 week, under a 16h light and 8hr dark regime at 20°C. A 3µl aliquot of the appropriate transcription mix was combined with 22 µl FES buffer (Scofield *et al*, 2005) making 25 µl/plant and was applied by gentle rubbing of the first leaf. The virus was allowed to develop for two weeks, during which the daylight temperature was increased to 25°C. The segments from the 3rd and 4th leaf were cut from the virus infected plants and were inoculated with powdery mildew isolate 98275. The phenotype was recorded 8 days post inoculation.

5.6 Single cell transient transformation assay and microscopy

Biolistic bombardment was performed as described in Yahiaoui *et al*, 2006. Leaves of the powdery mildew susceptible line Chancellor were bombarded with a 1:1 (wt/wt) mixture of pUbiGUS containing the GUS reporter and the PGY1 control vector containing the *Pm3CS* gene or the new *Pm3* alleles isolated from the FIGS-powdery mildew set of landraces. Leaf segments

were infected with *B. graminis* f.sp. *tritici* 4 hours after the bombardment. Staining for GUS activity was carried out 48 hours post inoculation. Fungal structures were subsequently stained with Coomassie blue. GUS expressing epidermal cells attacked by a single germinating spore were evaluated by transmission light microscopy. A susceptible interaction was characterized by a mature haustorium and elongating secondary hyphae. A resistant interaction was characterized by the presence of an appressorium. At least three independent experiments were carried out, each time counting at least 50 interactions. For domain swap experiment, the constructs were confirmed by sequencing. Staining for hypersensitive response was performed on leaf segments of resistant landraces using lacto phenol-trypan blue. The stained leaves were cleared in a chloral hydrate solution. In microscopy, host cells with haustorium and elongating secondary hyphae marked the successful penetration of fungus. LPTB is a histochemical indicator of cell death, so the blue colouration marks dead cells.

5.7 Semi-quantitative RT-PCR

Total RNA was extracted from leaves of 10 day old seedlings using TRIzol reagent (Invitrogen Life Technologies). For RT, 2 μg of total RNA was denatured at 70°C for 5 min in the presence of 0.06 μg of oligo$(dT)_{20}$ primers. Reverse transcription was done with the M-MLV reverse transcriptase kit (Promega). The reaction was inactivated at 70°C for 15 min. Aliquots of the reverse transcripts (2 µl from 1:8 dilution) were then amplified in a 25 µl PCR reaction, containing 0.2 mM specific oligonucleotide primers.

VI. Independent evolution of functional *Pm3* resistance genes in wild tetraploid wheat and domesticated bread wheat

Nabila Yahiaoui, Navreet Kaur and Beat Keller

1. Summary

The *Pm3* alleles of cultivated bread wheat confer gene for gene resistance to the powdery mildew fungus. They represent a particular case of plant disease resistance gene evolution because of their recent origin and possible evolution after formation of hexaploid wheat. The *Pm3* locus is conserved in tetraploid wheat, allowing a comparative evolutionary study of the same resistance locus in a domesticated species and one of its wild ancestors. We have identified 61 *Pm3* allelic sequences from wild and domesticated tetraploid wheat subspecies. The *Pm3* sequences corresponded to 24 different haplotypes. They showed low sequence diversity, differing by few polymorphic sequence blocks that were further reshuffled between alleles by gene conversion and recombination. Polymorphic sequence blocks are different from the ones found in functional *Pm3* alleles of hexaploid wheat, indicating an independent evolution of the *Pm3* loci in the two species. A new functional gene was identified in a wild wheat accession from Syria. This gene, *Pm3k*, conferred intermediate race-specific resistance to powdery mildew and consists of a mosaic of gene segments derived from non-functional alleles. This demonstrates that *Pm3*-based resistance exists at low frequency in wild tetraploid wheat and that the evolution of functional resistance genes occurred independently in wild tetraploid and in bread wheat. *Pm3* sequence variability and geographical distribution indicated that diversity was higher in wild emmer wheat from the Levant area compared

to the accessions from Turkey. Further screens for *Pm3* functional genes in wild wheat should therefore focus on accessions from the Levant region.

2. Introduction

Plant disease resistance genes (*R* genes) are a major component of the plant response to pathogen attacks. A majority of *R* genes encode proteins with a nucleotide-binding (NB) and a leucine-rich repeat (LRR) domain associated at the N-terminus either with a Toll-interleukin receptor-like (TIR) domain or a coiled-coil (CC) domain. R proteins directly or indirectly perceive pathogen emitted effector molecules and trigger a resistance reaction mainly based on a hypersensitive response leading to cell death (Jones and Dangl, 2006).

At the molecular level, plant *R* genes show complex patterns of variation among different accessions/cultivars. To understand this diversity, *R* genes have been studied in the wild model plant species Arabidopsis. There, population genetic analysis at the *RPM1*, *RPS2*, *RPS5* and *RPP13* loci revealed high levels of polymorphism between alleles (*RPP13*, *RPS2*) and distinct resistant and susceptible haplotypes (*RPM1*, *RPS5*, *RPS2*) indicating that ancient genetic variation has been maintained by balancing selection (Bakker *et al*, 2006; Bergelson *et al*, 2001; Caicedo *et al*, 1999; Rose *et al*, 2004; Stahl *et al*, 1999). More recently, a genome wide survey of *R* gene polymorphism across 96 Arabidopsis accessions also identified a number of loci showing low levels of polymorphism suggesting that alleles at *R* gene loci can be generated and maintained for short periods of time (Bakker *et al*, 2006).

In contrast to the studies in the wild species Arabidopsis, there is only limited knowledge on the comparative evolution of *R* genes in domesticated species and their wild relatives. In the few studies available, the analysis of evolution and natural variation mainly focused on *R* genes that had been very recently introgressed from wild relatives into cultivated species. These genes showed basically no polymorphism in crop plants and were found well conserved in wild donor accessions and related species. The *Cf* genes found in the *Solanum* sp. confer resistance to *Cladosporium fulvum* and represent the best studied case of a comparative analysis in wild and domesticated species. The *Cf9* gene and a functional chimeric homolog (*9DC*) were highly conserved and occurred frequently along the geographical range of the donor species *Lycopersicon pimpinellifolium* (Van der Hoorn *et al*, 2001). Highly similar functional *Cf4* and *Cf9* orthologs were identified in diverged *Lycopersicon* wild species suggesting that the origin of these genes predated *Lycopersicon* speciation (Kruijt *et al*, 2005). In contrast, in a recent study in the lettuce/*Bremia lactuca* pathosystem, only one active *Dm3* allele was discovered in more than 1000 wild accessions suggesting a possibly recent evolution of the gene (Kuang *et al*, 2006). This *Dm3* allele had the same specificity and only three amino acid changes compared to the *Dm3* resistance gene against *Bremia lactucae* from cultivated lettuce.

R genes evolve through a variety of molecular mechanisms: point mutations, unequal crossing over, gene conversion and recombination, illegitimate recombination and insertion/deletions all contribute to variability (Kuang *et al*, 2004; Michelmore and Meyers, 1998; Wicker *et al*, 2007). Sequence variability in *R* genes/alleles was mostly found in the LRR region which was shown to play a major role in pathogen recognition specificity (e.g. Ellis *et al*, 1999; Shen *et al*, 2003; Yahiaoui *et al*, 2006).

The *Pm3* gene occurs in seven different alleles (*Pm3a* to *Pm3g*) which encode CC-NB-LRR proteins that confer race-specific resistance to *Blumeria graminis* f. sp. *tritic*i, the wheat powdery mildew fungus (Srichumpa *et al*, 2005; Yahiaoui *et al*, 2004; Yahiaoui *et al*, 2006). The seven resistance alleles showed low levels of sequence divergence but functional diversity. They differ from a widespread susceptible allele (*Pm3CS)* by a few point mutations in the LRR region or by few tracks of sequence changes possibly derived from gene conversion. The *Pm3* locus is present in wild emmer wheat, *Triticum turgidum* subsp. *dicoccoides*, the progenitor of cultivated tetraploid and hexaploid wheat. The susceptible *Pm3CS* allele was detected in wild emmer wheat accessions from areas close to the Karacadag mountains in southeastern Turkey (Yahiaoui *et al*, 2006), a region proposed to be the site of wheat domestication (Luo *et al* 2007; Ozkan *et al*, 2002; Ozkan *et al*, 2005). This led to the hypothesis that bread wheat *Pm3* resistance alleles evolved from the ancestral *Pm3CS* sequence after wheat domestication 10,000 years ago.

The comparative analysis of disease resistance genes which have evolved in parallel in wild and domesticated species allows the study of molecular evolutionary mechanisms creating functional diversity in resistance. In particular, it allows to determine whether resistance alleles (i) derive from a functional ancestor gene or (ii) independently evolved the resistance function. The presence of the *Pm3* locus in both the wild and cultivated wheat thus provides a unique opportunity to study the functional evolution of this locus in both species.

3. Results

3.1 Isolation of *Pm3* alleles from tetraploid wheat species

Wild emmer is subdivided into two populations (Nevo and Beiles, 1989, Ozkan *et al*, 2005): the Northern one in Turkey, Iran, Irak and the Southern one in the Levant region (Lebanon, Jordan, Israel, Syria). To study the diversity of the *Pm3* gene in tetraploid wheat, we analysed a total of 179 accessions derived from different sources. A set of 124 accessions was from a collection of *T. turgidum* wild and cultivated subspecies that was assembled by Ozkan *et al* (2002, 2005). This collection covers both the Northern population (Turkey, 18 accessions) and the Southern population of emmer wheat (69 accessions) (Appendix 9.3). In addition, it includes domesticated tetraploid wheat from different, mostly European, countries (*T. dicoccum* and *T. durum*, 37 accessions). We also included 49 accessions from the NSGC (National Small Grain Collection), comprising (i) 27 wild wheat accessions specifically from the Karacadag range in Southeastern Turkey from which domesticated wheat might have originated and (ii) *T. durum* lines mostly from Turkey and Ethiopia. In total, we screened for their *Pm3* haplotype 130 *T. dicoccoides*, 14 *T. dicoccum* and 35 *T. durum* accessions from different countries (Appendix 9.6). We first analysed the accessions with an STS marker which is highly correlated with the presence of any *Pm3* allele (Tommasini *et al*, 2006; Yahiaoui *et al*, 2006). This marker amplifies a 900 bp fragment of the 5' non coding region of *Pm3*. A total of 63 *T. dicoccoides* and 6 *T. durum* accessions showed amplification of this marker. The majority of these accessions are from the Asian part of Turkey or from the Levant area (Israel, Lebanon and Syria). None of the European accessions showed amplification of the *Pm3* marker. Full length coding sequences were obtained from 19 *T.*

dicoccoides and from 2 *T. durum* accessions. Failure to amplify *Pm3* genes from the remaining accessions could be due to deletion of the gene or low sequence homology at primer binding sites. In our analysis we also included 40 sequences from a previous screening of 201 accessions of wild and cultivated tetraploid wheat subspecies (Yahiaoui *et al*, 2006, appendix 9.6). Finally, a total of 61 *Pm3* sequences from tetraploid wheat were available, among them 47 sequences from wild tetraploid *T. dicoccoides* and 14 from domesticated *T. dicoccum* and *T. durum.*

3.2 Haplotype diversity of the *Pm3* alleles from tetraploid wheat

All *Pm3* alleles from tetraploid wheat had a size of 4442 bp, comprising two exons of 4156 bp and 86 bp and an intron of 200 bp. They did not contain any indels compared to the *Pm3CS* reference sequence previously described as a susceptible *Pm3* allele in cultivated bread wheat (Yahiaoui *et al*, 2006). Sequence alignment and analysis indicated that the *Pm3* genes from the 61 tetraploid wheat accessions corresponded to 24 different sequences (haplotypes, Appendix 9.5 and Figure 6.1). Almost half of the sequences (28 out of 61, 46 %) belonged to three haplotypes (H1, H2, and H16, Appendix 9.5). Haplotypes H1 and H2 only differ by one base pair (bp1138) resulting in one amino acid change (L_{380} to V_{380}) in the NBS region (Figure 6.1). Accessions carrying *Pm3* sequences of the H1 haplotype comprise both wild *T. turgidum* subsp *dicoccoides* from Turkey and cultivated *T. turgidum* subsp. *dicoccum* from Irak, Iran and Russia whereas those of the H2 haplotype were all wild wheat accessions from the Karacadag region in Turkey. One *T. dicoccoides* accession from Iran (IG113302, Appendix 9.5) defines a haplotype (H4) which only differs from haplotype H1 by a silent mutation at bp 1119 in the NB encoding region. The third major haplotype H16 consists of sequences identical to *Pm3CS*. Four

haplotypes were found (H17 (previously H16* in Yahiaoui *et al*, 2006), H18, H19 and H23) which only show very few polymorphic residues compared to H16 (*Pm3CS*). Finally, a total of fourteen haplotypes were identified (58 % of the total number) that were represented by single accessions.

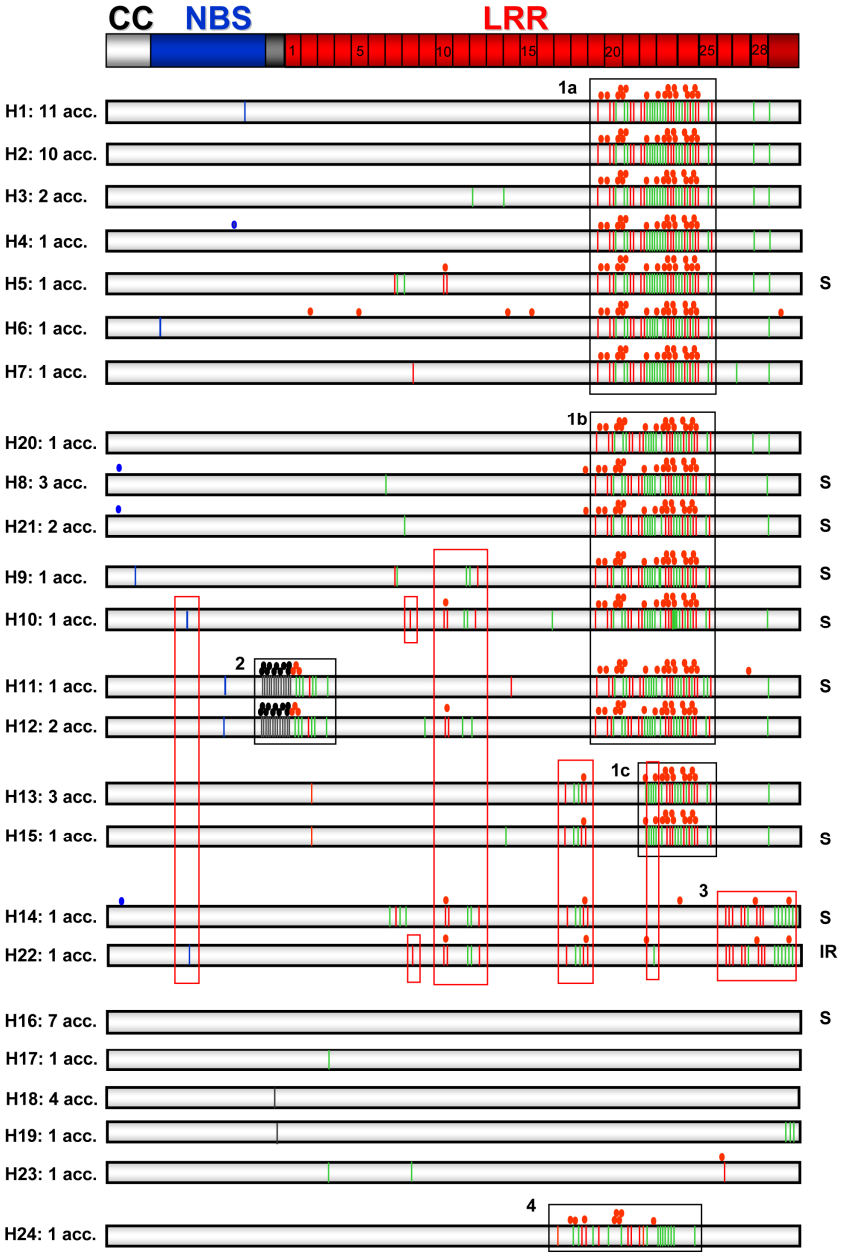

Figure 6.1. Schematic representation of the different protein sequences encoded by tetraploid wheat *Pm3* alleles.

From 61 sequences, 24 different haplotypes (H1 to H24) were identified based on their sequence divergence. The number of accessions corresponding to each haplotype is indicated on the left. The different domains encoded by *Pm3* genes are represented at the top. CC, coiled-coil (light grey box); NB (blue box), nucleotide-binding domain; spacer region (grey box); LRR (red boxes), leucine-rich repeats. LRR numbers are indicated. Colored bars in PM3 proteins represent polymorphic amino acids compared to the PM3CS protein, which was chosen as a reference sequence. Blue bars are polymorphic amino acids in the NB domain, black bars in the spacer region. Green bars represent amino acid replacements in structural LRR residues and red bars amino acid changes in the X solvent exposed residues of the XXLXLXX motif of LRR repeats. Colored dots represent synonymous mutations. Haplotypes were grouped based on sequence homology. Polymorphic sequence blocks compared to the H16 (PM3CS) sequence are boxed in black and numbered. Block 1a and 1b differ by 2 amino acids, the H6, H9 and H10 sequences show 1 to 2 additional polymorphisms. Boxes in red indicate gene segments found in susceptible alleles as well as in active resistance allele *Pm3k* (H22). Results of the functional assay of selected genes are indicated on the right. S, susceptible allele; IR, intermediate resistance.

Table 6.1. Haplotype and nucleotide diversity in different coding regions of *Pm3* genes from all tetraploid wheat species analysed.

	Haplotypes	Number of sites	Segregating sites	π total	π S	π NS
Entire gene excl. intron (1-4242 bp)	24	4242	185	0.87	0.98	0.83
CC-NB region (1-1515 bp)	9	1515	10	0.06	0.09	0.05
Spacer (1516-1734 bp)	3	219	31	1.5	2.2	1.3
LRR (1735-4242 bp)	22	2508	143	1.4	1.5	1.4

Sixty one sequences were analysed. The nucleotide diversity π is presented as the average number of pairwise nucleotide differences per 100 bp between DNA sequences. S, synonymous, NS, nonsynonymous.

The nucleotide diversity was analysed for the whole coding sequence of the *Pm3* genes and specifically for the regions encoding the distinct *Pm3* protein domains (CC-NB domain, spacer and LRR domain, Table 6.1). This indicated that most of the haplotype and sequence diversity between *Pm3* sequences was present in the LRR encoding region. There, 22 different haplotypes were identified compared to only 9 and 3 in the CC-NB region and spacer regions, respectively. The very low level of polymorphism in the CC-NB region is illustrated by the scarce polymorphic residues shown in Figure 6.1 in this region and low values of synonymous and non synonymous nucleotide diversity. In contrast, in the spacer and LRR regions, values of nucleotide diversity (π) were higher (Table 6.1). In these regions, polymorphic sequence blocks of various sizes were found between *Pm3* haplotypes (Figure 6.1). The overall value of nucleotide diversity (π total= 0.0085, Table 6.1) for the entire tetraploid *Pm3* gene sequences was lower than what was previously described in other *R* gene studies in wild species, such as for the *RPP13* allelic series in *Arabidopsis thaliana* (π total= 0.045, Rose *et al*, 2004). This suggests a relatively recent divergence of these *Pm3* genes in the tetraploid gene pool.

Haplotypes H1 to H12 and H20/21 all differ from the remaining haplotypes by the presence of a highly polymorphic sequence block between bp 3220 and bp 3843 (block 1, LRR19-LRR25, Figure 6.1). This sequence block of 624 bp size shows a total of 67 polymorphic bases consisting of 49 non synonymous (replacement) mutations and 18 synonymous (silent) mutations. Within this block, some sequences showed small differences of 2 to 4 amino acids (e.g. block 1a and 1b, Figure 6.1). Block 1 is only partially present in H13 and H15 (block 1c, Figure 6.1). In the spacer/ first LRRs region, haplotypes H11 and H12 differ from all other haplotypes by a sequence block of 353 bp

that includes the first three LRRs (block 2, Figure 6.1). The haplotypes H14 and H22 share a common and specific polymorphic sequence block at the 3' end of the coding sequence (block 3, Figure 6.1) whereas the *T. durum* haplotype H24 shows a polymorphic sequence block (block 4, Figure 6.1) which also contains fragments of block 1 and amino acids present in H14 and H22. Except for *Pm3CS* (H16), none of the *Pm3* haplotypes from tetraploid wheat corresponds to known *Pm3* alleles from hexaploid bread wheat. The polymorphic sequence blocks (compared to the *Pm3CS* reference sequence) were not found as such in *Pm3* resistance alleles from bread wheat. Only small fragments of blocks 1 and 2 were identified in the *Pm3a* and *Pm3c* sequences, respectively (Yahiaoui *et al*, 2006).

3.3 Race-specific resistance conferred by a *Pm3* allele from tetraploid wheat

No functional *Pm3* resistance alleles have been identified in wild wheat species (McIntosh *et al*, 2003, http://wheat.pw.usda.gov/ggpages/wgc/2006upd.html). To test if any of the newly identified *Pm3* alleles from wild wheat might be functional, we carried out leaf infections at the seedling stage with three different powdery mildew races (Appendix 9.5). The powdery mildew isolates differed in their virulence / avirulence pattern to known *Pm3* resistance alleles in hexaploid wheat: Isolate 96224 is avirulent on all functional *Pm3* alleles (except for an intermediate susceptible reaction with *Pm3g*) whereas isolates 97019 (*AvrPm3a, AvrPm3b, AvrPm3c, avrPm3d, AvrPm3e, AvrPm3f, avrPm3g*) and DB Asosan (*avrPm3a, avrPm3b, avrPm3c, AvrPm3d, avrPm3e, avrPm3f, avrPm3g*) show differential reactions on lines with the different *Pm3* alleles. The six haplotypes H4, H12, H19, H20, H23 and H24 were represented by one or two accessions and all of them were susceptible to the three powdery mildew isolates. Within the H1 and

H2, H3, H8, H10, H13 and H16 haplotypes, accessions carrying identical *Pm3* sequences showed different patterns of resistance and susceptibility to the three isolates suggesting that the resistance observed in some of the accessions was not due to the *Pm3* gene present in these lines. Among these haplotypes, H16 corresponds to the *Pm3CS* allele which was previously shown to be non-functional against powdery mildew (Yahiaoui *et al*, 2006). Finally, 11 haplotypes were mostly represented by single accessions which showed complete resistance (H6, H7, H9, H11, H14, H15, H17, H18, H21, H22) or intermediate resistance (H5) to the three tested isolates (Appendix 9.5).

Nine *Pm3* genes (H5, H8, H9, H10, H11, H14, H15, H21, H22, Figure 6.1) were tested in a transient transformation assay (Schweizer *et al*, 1999; Yahiaoui *et al*, 2004). These genes were either from accessions showing full resistance or from accessions with different phenotypes. As a control for these experiments, we used the non functional allele *Pm3CS* (H16) (Yahiaoui *et al*, 2006). The coding sequences of the *Pm3* candidate genes or of the *Pm3CS* (H16) control were co-bombarded with a plasmid carrying the β-glucuronidase (GUS) reporter gene into leaf epidermal cells of the powdery mildew susceptible bread wheat line Chancellor. The leaf segments were subsequently infected with wheat powdery mildew isolate 96224 and the percentage of compatible (susceptible) interactions was determined. Eight of the tested *Pm3* genes (H4, H8, H9, H10, H11, H14, H15 and H21) were not functional against isolate 96224 (data not shown). Only one of the tested genes, H22 conferred resistance to isolate 96224 in the transient transformation assay (Figure 6.2a). In the control experiments using *Pm3CS*, 76% of the cells expressing the GUS reporter gene and attacked by one powdery mildew spore showed fully developed haustorial structures indicative of a compatible interaction (Figure 6.2a). When

H22 was used, a significant reduction of the percentage of compatible interactions (39% compatible interactions) was observed after infection with isolate 96224 (Figure 6.2a). The activity of H22 was also confirmed using isolate 97019 where a significant decrease in compatible interactions was observed (83 % of compatible interactions for the control, 52% for H22).

To check for race specificity of the resistance conferred by H22, we identified a powdery mildew isolate (97028) which is virulent on IG46439, the wild wheat accession carrying H22 (Figure 6.2b). When tested in the transient assay, H22 did not confer resistance to the virulent isolate 97028 (69% compatible interactions for H22, 74% for the control *Pm3CS*) demonstrating a race-specific activity of H22 (Figure 6.2a).

Interestingly, the H14 haplotype which is very close in sequence to H22 (Figure 6.1) was found to be non functional against isolate 96224 in the transient assay (Figure 6.2a). The two sequences differ by one amino acid in the NBS domain (V_{191} (H14) to F_{191} (H22)) and by eight residues in the LRR domain. Seven of these residues are also found in other alleles non functional against tested powdery mildew isolates, albeit in different sequence contexts, and only one polymorphic amino acid is specific to H22. Interestingly, this polymorphic amino acid (C_{1332}) is a solvent exposed residue within the conserved motif (LXXLXLXX) of LRR27. The new functional *Pm3* allele is named *Pm3k*. The resistance conferred in the transient assay by *Pm3k* (H22) is intermediate (between 61 and 48 % incompatible interactions). However, a phenotype of full resistance was observed on primary leaves of the IG46439 accession when infected with isolate 96224 (Figure 6.2b).

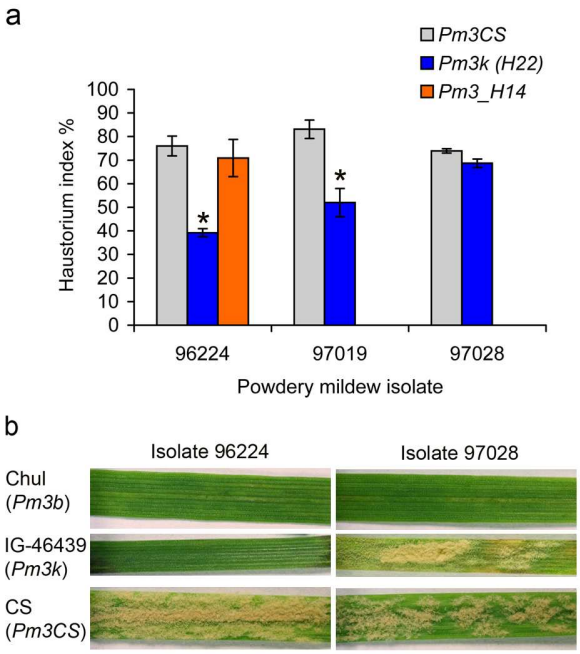

Figure 6.2. Functional analysis of the *Pm3k* gene (H22 haplotype) from the tetraploid *T. dicoccoides* accession IG46439.

(a) Results of the transient transformation assay of *Pm3k* (H22 haplotype) and *Pm3_TTD20* (H14 haplotype). The *Pm3* genes from tetraploid wheat were tested using a transient expression assay in epidermal cells of bread wheat *cv.* Chancellor. Genomic sequences of the candidate resistance genes and the susceptible control gene (*Pm3CS*) were under control of the 35SCaMV promoter. Cells expressing the GUS reporter gene were evaluated when attacked by a single mildew spore. The haustorium index, i.e. the percentage of compatible interactions, is indicated by the mean and the standard deviation of three independent experiments, each contributing at least 50 interactions. Powdery mildew isolates 96224, 97019 and 97028 were used. * indicates significant differences at P =0.05 compared to the control experiments. **(b)** Differential phenotypic reaction of wild wheat accession IG-46439 at 8 days after infection with the avirulent powdery mildew isolate 96224 and the virulent isolate 97028. Bread wheat lines Chul carrying the *Pm3b* resistance allele and Chinese Spring (*Pm3CS*) were used as controls. Chul is resistant and Chinese Spring (CS) is fully susceptible.

To check if the resistance conferred by *Pm3-IG46439 in planta* might be activated later than the 48h after infection assessed in the transient assay, we performed Trypan blue staining experiments of IG46439 infected with the avirulent isolate 96224. We observed that in 94% of the interactions, pathogen growth was blocked at the appressorium stage at 48hpi whereas haustoria were formed in 6% of the interactions. In a wheat accession with the strong *Pm3b* allele, there were no haustoria formed at 48hpi. Thus, *Pm3-IG46439* is a weaker allele which depends on reducing hyphal growth also at later stages than 48hpi as no pathogen growth was observed macroscopically after 7 days.

3.4 Phylogenetic analysis of the different *Pm3* haplotypes

A neighbour-joining phylogenetic tree based on nucleotide sequences of the *Pm3* allelic sequences was established (Appendix 9.4). Two main clades of *Pm3* allelic sequences were identified. However, interior branches within each clade were poorly supported. To better represent possible conflicting phylogenetic relationships between the *Pm3* allelic sequences, we inferred phylogenetic relationships between the 24 haplotype sequences using the split network method (Huson and Bryant, 2005).

The obtained neighbor-net (Figure 6.3) shows a separation between the haplotypes H1 to H10, H20 and H21 and haplotypes H16 (*Pm3CS*) to H19 (from hereafter these two groups will be defined as H1 type and H16 type). The haplotypes H11/12 and H13/H15 are more closely related to the H1 group whereas H14/H22 are more closely related to the H16 (*Pm3CS*) group. The H1 and H16 groups principally differ by the presence of the highly polymorphic sequence block 1 (between LRR19 and LRR25, Figure 6.1) which is also entirely or partially present in H11/12 and H13/H15 but is absent from H14 and H22 (where it is replaced by a *Pm3CS* type of sequence). In addition, all these sequences also

share common smaller blocks of sequence polymorphisms (Figure 6.1) which were possibly integrated and exchanged by recombination/gene conversion events. The presence of a series of parallel edges in the neighbour-net network between the different *Pm3* haplotypes (most particularly between H9/H10, H24, H22/H14 and H13/H15) is indicative of a complex evolutionary pattern which is probably due to sequence exchange by recombination/gene conversion between these genes.

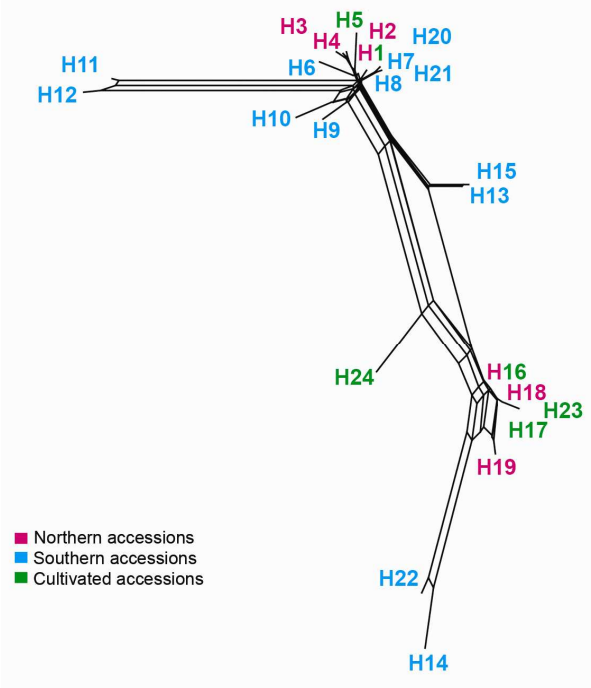

Figure6.3. Neighbour-net analysis obtained from the 24 haplotypes of *Pm3* alleles from tetraploid wheat species based on uncorrected *P*-distance. Several haplotypes are connected to each other by multiple pathways, forming an interconnected network and suggesting recombination/gene conversion.

Given the pattern of sequence polymorphism mostly based on clearly delimited sequence blocks and the recombination/gene conversion events leading to the actual diversity of *Pm3* alleles in tetraploid wheat, it is difficult to estimate a divergence time between these alleles. The major polymorphic sequence block between H1 and H16 sequence types carries a conserved number of synonymous mutations that can be used to calculate the

age of its divergence. Using the number of synonymous mutations in this block, a *p*-distance value (Nei and Gojobori, 1986) ds = 0.119 synonymous mutations per synonymous site was obtained. Using the grass *Adh* gene average substitution rate of $6.5x10^{-9}$ substitutions per synonymous site per year (Gaut *et al*, 1996), the divergence time for this sequence block is estimated to be 4.5 MYA . This indicates that the sequence blocks diverged before the hybridization event leading to the creation of tetraploid wheat species, 0.5 MYA (Huang *et al* 2002). Outside of the polymorphic blocks, very few sparsely distributed silent mutations were present in the *Pm3* sequences (Figure 6.1). This would be in accordance with a recent divergence after the hybridization creating tetraploid wheat.

3.5 Geographical and genetic differentiation of *Pm3* haplotypes in different sub populations of tetraploid wheat

To analyse the level of *Pm3* allelic diversity among different tetraploid wheat subpopulations, we estimated the haplotype diversity and sequence polymorphism from cultivated and wild wheat accessions and specifically from Northern wild wheat accessions (Turkey, Iran) and Southern wild wheat accessions (Israel, Jordan, Lebanon, Syria). DNA polymorphism analysis revealed a higher haplotype and nucleotide diversity for sequences from wild wheat accessions compared to cultivated wheat accessions (Table 6.2a). However, the smaller sample size of cultivated wheat sequences might introduce a bias in the analysis. In the comparison between Northern and Southern accessions of *T. dicoccoides*, we found that the *Pm3* haplotype and nucleotide diversity were higher in accessions of *T. dicoccoides* from the Southern area as compared to the accessions from Turkey.

Table6.2a. DNA polymorphism between *Pm3* sequences from different subpopulations of tetraploid wheat.

Population	Number of accessions	Number of haplotypes	Hd (haplotype diversity ± SD)	Polymorphic sites	Nucleotide diversity π (% ± SD)
All accessions	61	24	0.92 ± 0.02	190	0.84 ± 0.08
Cultivated	14	6	0.68 ± 0.02	86	0.74 ± 0.14
Wild accessions	47	20	0.93 ± 0.05	176	0.87 ± 0.1
Northern accessions (W)	26	7	0.80 ± 0.05	74	0.72 ± 0.07
Southern accessions (W)	21	13	0.94 ± 0.03	168	0.92 ± 0.2

The analysis was performed using the DNaSP program on whole gene sequences (intron included). SD, standard deviation. Northern accessions are from Turkey and Iran, Southern accessions are from Israel, Jordan, Lebanon, Syria.

To test for genetic differentiation between *Pm3* haplotypes from different subpopulations of tetraploid wheat accessions, a statistical analysis was performed (Table 6.2b). Two types of statistical values were calculated: Hst for the haplotype diversity and the Kst* and Snn sequence-based statistics for the nucleotide diversity (Hudson *et al*, 1992, Hudson 2000). The most highly significant probabilities of genetic differentiation based on haplotype and nucleotide diversity were found between wild vs. cultivated accessions, Northern vs. Southern wild wheat accessions and cultivated vs. wild Southern accessions. The cultivated vs. wild Northern accessions showed a lower statistical significance for genetic differentiation based on *Pm3* sequences. This is consistent with the idea that cultivated domesticated wheat species or subspecies might have originated from the Northern pool of wild wheat (Luo *et al*, 2007; Ozkan *et al* 2005), more particularly from

the Karacadag region in South Eastern Turkey which is represented by several accessions in our study (Appendix 9.5).

Table 6.2b. Analysis of the genetic differentiation between *Pm3* alleles from different subpopulations of tetraploid wheat accessions.

Accessions	Hd	Hst	Kt	Kst*	Snn
Wild (W.) *vs.* cultivated (n_1=47, n_2= 14)	0.923	0.05 (0.0)***	37.37	0.0089 (0.13)ns	0.81 (0.0) ***
W. Northern *vs.* Southern (n_1=26, n_2=21)	0.929	0.07 (0.0)***	38.85	0.11 (0.000)***	0.98 (0.0)***
Cultivated *vs.* W. Northern (n_1=14, n_2=26)	0.836	0.09 (0.001)**	31.64	0.0085 (0.255) ns	0.73 (0.001)**
Cultivated *vs.* W. Southern (n_1=14, n_2=21)	0.931	0.096 (0.0)***	39.68	0.083 (0.001)**	0.97 (0.0)***

Values for mean haplotype diversity (Hd), mean nucleotide differences (Kt) and for haplotype (*Hst*) and sequence based statistics (*Kst**, *Snn*) are calculated based on Hudson *et al* (1992) and Hudson (2000). Probabilities (between brackets) were calculated using a permutation test with 1000 replicates. ns, not significant; ** significant $0.001<P<0.01$; *** highly significant $0.000<P<0.001$. W, wild wheat accessions.

4. Discussion

We have identified and analysed a large set of 61 *Pm3* allelic sequences corresponding to 24 different haplotypes found in tetraploid wheat subspecies. *Pm3k*, a new resistance allele of the *Pm3* gene in tetraploid wheat was identified, shedding light on the evolutionary events creating active powdery mildew resistance genes in wild and domesticated wheat. The analysis of phylogenetic relationships, haplotype diversity and geographical distribution of the *Pm3* genes revealed that *Pm3* evolution paralleled the evolution of wild and domesticated wheat species.

4.1 Comparative evolutionary analysis of *Pm3* alleles in wild tetraploid wheat vs. hexaploid wheat

Although resistance to powdery mildew was quite frequent in wild tetraploid wheat, we found that it was mostly due to other loci than *Pm3*. In our screen for *Pm3*-based resistance, we have used European powdery mildew strains isolated from bread wheat and combining differential virulence and avirulence on known *Pm3* resistance alleles. One functional *R* gene against these isolates was identified, but it is possible that some of the other *Pm3* alleles from wild wheat are active against specific local isolates from wild wheat collection areas.

The low frequency of *Pm3*-based resistance in wild emmer is similar to the situation in lettuce where the *Dm3* resistance gene against *Bremia lactucae* was rare in natural populations of the wild lettuce *Lactuca serriola* (Kuang *et al*, 2006). It was postulated that the low frequency of *Dm3* was either due to recent evolution of the locus, to deletion of the gene or to high variation in sequence which made its detection difficult. In the case of *Pm3*, the gene was present in a large number of accessions and *Pm3* sequences were conserved enough to be identified. Therefore, low frequency of *Pm3*-based resistance in wild emmer could be due to an independent evolution of active resistance genes in the tetraploid vs. hexaploid wheat gene pools. The situation at the *Dm3* and *Pm3* loci differs from studies of natural variation of *R* genes in Arabidopsis. There, the frequency of studied functional *R* genes was high and most of the *R* gene orthologs or alleles were well conserved in function and of ancient origin (Bergelson *et al*, 2001; Rose *et al*, 2004; Bakker *et al*, 2006).

Until now only one *Pm3* sequence, the *Pm3CS* (H16) allele, was found identical between bread wheat and tetraploid wheat. In bread wheat, the *Pm3* resistance alleles diverged

from the *Pm3CS* template either by few specific point mutations or by gene conversion events introducing polymorphic sequence blocks which are different from the ones found in tetraploid wheat (Yahiaoui *et al*, 2006, and this work). The functional *Pm3k* allele of wild emmer shows polymorphic sequence blocks which are not found in known bread wheat resistance alleles. This indicates that resistance conferred by this gene independently evolved within the tetraploid gene pool.

4.2 *Pm3k* consists of building blocks from non-functional alleles

The functional *Pm3k* (H22) sequence is a chimeric arrangement of blocks from alleles non-functional against tested powdery mildew isolates. A mosaic pattern of conserved sequences is found among the flax rust resistance *L* alleles (Ellis *et al*, 1999) but this sequence exchange occurred between functional alleles. A reshuffling of non functional sequences to generate resistance genes was described for *Cf* genes which originate from a combination of sequences from paralogous genes (Parniske *et al* 1997). Thus, the structure of *Pm3k* supports the proposed hypothesis of recycling polymorphism where non functional sequences serve as a reservoir of variation to generate functional genes (Holub, 2001).

The LRR domain and particularly variable solvent exposed residues are proposed to be responsible for the specificity of NB-LRR proteins (Ellis *et al*, 1999, Jones and Jones 1997) and were shown to play an important role for *Pm3* specificity of resistance (Yahiaoui *et al*, 2006). The LRR domain also plays an important function in the negative or positive regulation of R proteins through intramolecular interactions with the NB domain (Moffett *et al*, 2002; Rairdan and Moffett, 2006). The *Pm3k* (H22) sequence carried only a single polymorphism not found in any susceptible allele (C_{1332}), which is a

solvent exposed residue of LRR27. Interestingly, in hexaploid bread wheat, one amino acid change in a solvent exposed residue of LRR27 (E_{1334} to V_{1334}) was sufficient to convert the susceptible *Pm3CS* into a functional resistance allele (Yahiaoui *et al*, 2006). This suggests that the function of *Pm3k* H22 could be due to the C_{1332} specific polymorphism in LRR27 and points out to the importance of this particular region of the LRR for PM3 protein function. However, it cannot be excluded that the combination in H22 of sequence motifs shared with the other emmer wheat *Pm3* proteins also plays a role in *Pm3k* function.

It is difficult to determine if *Pm3k* has a different spectrum of race specificity compared to other *Pm3* alleles. Accession IG46439 was resistant to several tested isolates (Kaur and Keller, unpublished data) and it is not excluded that other genes present in IG46439 contribute to the resistance phenotype. Based on transient assay data, *Pm3k* conferred intermediate resistance to isolates avirulent to most *Pm3* alleles of bread wheat but not to isolate 97028 which is virulent on all *Pm3* alleles except for *Pm3a* and *Pm3b*. Race specificity of the *Pm3k* resistance is therefore certainly different from the one of *Pm3a* and *Pm3b*.

4.3 Timing and mechanisms of evolution of tetraploid wheat *Pm3* alleles

Phylogenetic analysis of *Pm3* haplotypes from tetraploid wheat identified two main clades which differed mostly by one sequence block. This sequence block is of ancient origin with a divergence time corresponding to the divergence of ancestral wheat diploid species (Dvorak *et al*, 2006; Huang *et al*, 2002). In hexaploid bread wheat *Pm3* alleles, no synonymous mutations were found outside of clearly delimited polymorphic sequence blocks (Yahiaoui *et al*, 2006) leading to the hypothesis of a very recent divergence

10,000 years ago. In tetraploid wheat, few synonymous mutations were found outside of the polymorphic blocks. This likely reflects a relatively more ancient divergence compared to bread wheat alleles and is compatible with the estimated time of the first hybridization event at the origin of tetraploid wheat, less than 0.5 MYA (Huang *et al*, 2002).

Relationships between *Pm3* sequences are obscured by the low level of polymorphism and by recombination/gene conversion events involving the polymorphic segments. The *Pm3* alleles from tetraploid wheat show few polymorphisms which are found specifically in one haplotype only (1.5 unique nucleotide polymorphism per sequence). Most polymorphic sequences were found at least twice among the different haplotypes indicating frequent sequence exchange between alleles. Sequence exchange by gene conversion and/or recombination is one of the major mechanisms of *R* gene evolution (Kuang *et al*, 2004; Mondragon Palomino and Gaut, 2005) and it is also the main mechanism of *Pm3* evolution in tetraploid wheat.

4.4 The haplotype distribution and the geographical differentiation of *Pm3* alleles from tetraploid wheat provide insight in the history of wheat evolution

Among the 3 main tetraploid gene pools (wild *T. dicoccoides*, domesticated *T. dicoccum* and *T. durum*) that we have screened for their *Pm3* haplotype, most *Pm3* alleles were detected in wild *T. dicoccoides* accessions (43% of screened accessions compared to 15 and 13% for *T. dicoccum* and *T. durum* respectively). The wild gene pool is therefore enriched in *Pm3* sequences compared to the domesticated emmer.

In our screen, the three most frequent *Pm3* haplotypes were from Turkey, Iran and Irak (H1, H2 and H16). Two of these haplotypes comprise both wild and domesticated emmer accessions and therefore possibly identify the two main *Pm3* sequences that were

transmitted through domestication to the cultivated gene pool. The H16 haplotype is present in cultivated hexaploid wheat (Yahiaoui *et al*, 2006). It is specifically found in wild wheat accessions from Turkey (from or close to the Karacadag region), and closely related variants were either in wild emmer from the Karacadag region (H18, H19) or in domesticated emmer (H17, H23). Several studies have found that domesticated wheat is most closely related to the Northern gene pool of wild emmer wheat (Luo *et al* 2007; Ozkan *et al*, 2002; Ozkan *et al* 2005). The analysis of *Pm3* genetic differentiation also showed a significant differentiation between domesticated wheat accessions and wild wheat accessions from the Southern pool compared to accessions from the Northern pool. Our results are therefore in accordance with the hypothesis that domesticated wheat originates from the Northern pool.

A higher genetic diversity was described for the Southern population which is therefore proposed to be an important source for wheat improvement (Luo *et al*, 2007; Nevo, 1998). In agreement with this, our data on *Pm3* haplotype and nucleotide diversity indicate a higher diversity among accessions from the Southern pool. This could be due to more diverse eco-geographical conditions in in this region compared to the sampled areas of the Northern pool. The new resistance allele *Pm3k* was found in an accession from the Southern pool. This suggests that additional functional alleles can be found in accessions from this area and that further screenings should be made on Southern populations growing under specific and diverse ecological conditions.

The identification and the conservation of the *Pm3* locus at two different ploidy levels in the wheat gene pool and its presence in wild relatives and in domesticated species allowed to correlate and to link the evolution of this gene to the history of wheat

evolution and domestication. In terms of exploitation of the tetraploid gene pool to identify new functional *Pm3* resistance alleles our work shows that although this resistance is not very frequent, it is possible to identify functional *Pm3* variants in the tetraploid wheat gene pool. Partial resistance conferred by the gene we identified could be of agricultural interest as it would potentially induce less pressure on the pathogen populations and would therefore be of more durable use.

5. Experimental procedures

5.1 Plant material and wheat powdery mildew isolates

Accessions used for haplotype analysis and *Pm3* gene amplification were obtained from the Max-Planck-Institute for Plant Breeding Research (F. Salamini), J. David, (INRA Montpellier, France), M. Feldman (Weizmann Institute of Science, Israel), and from stock centers at IPK Gatersleben (Germany) and USDA National Small Grains Research Facility (USA). We use here the abbreviated forms *T. dicoccoides* for wild *Triticum turgidum subps. dicoccoides*, *T. dicoccum* for domesticated hulled emmer *T. turgidum subps. dicoccum* and *T. durum* for domesticated free threshing hard wheat (*T. turgidum subps. durum*). Wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*) isolates were provided by P. Streckeisen, Forschungsanstalt Agroscope Reckenholz-Tänikon, Switzerland and by D. Barloy, INRA Rennes and were maintained on wheat cv. Kanzler by weekly transfer to fresh plants.

5.2 *Pm3* haplotype analyses

Isolation of genomic DNA, PCR amplification and analysis were performed as previously described (Srichumpa *et al*, 2005). *Pm3* haplotype analysis was performed by sequence-

tagged-site (STS) marker analysis with primer pair UP3B (5'TGGTTGCACAGACAATCC3') and UP1A (5'GAAACCCGGCATAAGGAG3').

5.3 Cloning of *Pm3* alleles and test gene constructs

PCR amplification of the *Pm3* genes was carried out with the *Pfu*Ultra high-fidelity DNA polymerase (Stratagene, http://www.stratagene.com/) using a nested PCR strategy with primers UP6 (5' GGCACAGACAAAGCTCTG 3') and N3SP3RP (5' ACAATCAGGGATCAGGCC 3') in a first step and primers BamH1_1 (5'TTAATTGGATCCCCAATGGCAGAGCGGGTGGTC') and Sal1_1 (5'TATATAGTCGACGCTTCAGCTCCGGCAGGCCTG3') in a second step as previously described (Srichumpa *et al*, 2005). For *Pm3* alleles that were found as unique sequences in tetraploid wheat or that were tested in the transient assay, two independent PCR reactions were carried out. Amplified fragments were either sequenced as PCR products or cloned into the multiple cloning site of vector PGY1 (Schweizer *et al*, 1999) between a 540-bp fragment of the 35SCaMV promoter and the 35SCaMV terminator. Two clones per PCR reaction were sequenced. DNA sequencing was carried out on Applied Biosystems Capillary Sequencer model 3730 (http://www.appliedbiosystems.com/).

5.4 Single cell transient transformation assay

Biolistic bombardment was performed as described in Yahiaoui *et al*, 2004 and modified as in Douchkov *et al* (2005). Leaves of the powdery mildew susceptible line Chancellor were bombarded with a 1:1 (wt/wt) mixture of pUbiGUS containing the GUS reporter and the PGY1 control vector containing the *Pm3CS* gene (Yahiaoui *et al*, 2004) or the test gene constructs from tetraploid wheat. Leaf segments were infected with wheat

powdery mildew 4 hours after bombardment. Staining for GUS activity was carried out 48 hours post inoculation. Fungal structures were then stained with Coomassie blue. GUS expressing epidermal cells attacked by a single germinating spore were evaluated by transmission light microscopy. A compatible (susceptible) interaction was characterized by a mature haustorium and elongating secondary hyphae. An incompatible (resistance) interaction was characterized by the presence of an appressorium. A Student's two-tailed t test was performed as a statistical validation of the results.

5.5 Sequence analysis

Sequence assembly was performed using the Gap4 program of the Staden Package (http://staden.sourceforge.net/). The ClustalX software (Thompson *et al*, 1997) was used for sequences alignments that were visualized in the program Genedoc (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html). The different R protein domains (CC-NBS, LRR, structural LRR residues and solvent-exposed LRR residues in the XXLXLXX motif) were chosen according to Meyers *et al* (2003). Divergence time was estimated using the formula $r = \pi_s/4T$ were r is the divergence rate (the grass *Adh* gene substitution rate of $6.5\ 10^{-9}$ substitutions per synonymous site per year (Gaut *et al*, 1996) was used), π the synonymous nucleotide diversity and T the divergence time. Sequences of the *Pm3* genes from tetraploid wheat *Pm3_H1* to *Pm3_H15* and *Pm3_H17* to *Pm3_H24* have been deposited in the Genebank database under accession numbers EU192106 up to EU192128.

5.6 Phylogenetic and genetic differentiation analysis

The neighbour-net phylogenetic analysis was performed with the Splitstree program, based on uncorrected *P*-distance. Analysis of polymorphism data was performed with

DnaSP version 4.0 (Rozas *et al*, 2003). Nucleotide diversity was estimated for synonymous and nonsynonymous sites as π (Tajima, 1983). The DnaSP version 4.0 software (Rozas *et al*, 2003) was also used for the statistical analysis of genetic differentiation.

VII. General discussion

1. Targeted screening of wheat genetic collections for *Pm3* allele mining

As resistance may be lost with rapid emergence of new pathogen strains, it becomes a continuous task to identify new resistance genes and to transfer these genes into common wheat if they are present in wild relatives or related species. In the past, wild species have been replaced by landraces and traditional varieties which were further substituted by genetically less diverse modern cultivars. This has imposed genetic bottlenecks resulting in depletion of genetic diversity from the breeding germplasm. It is also well known that genetic uniformity can render the crop vulnerable to pathogens, while genetic diversity enables improvement in productivity and adaptation. The gene banks hold large collections of wild and cultivated diploid, tetraploid and hexaploid species which might carry the alleles needed for resistance and tolerance to the diseases, pests and harsh environments (Hoisington et al, 1999). The undisclosed but valuable allelic variants in this underexplored sustainable resource can enhance the crop improvement. However, the use of this diversity is hampered by the large number of accessions available and the limited resources which are at hand for phenotypic characterization of all these lines. Therefore, it is necessary to develop strategies to assemble focused sets of material for specific traits based on rational criteria for selection of the lines. The concept of a core collection is frequently proposed as a strategy to work with fewer accessions that would represent the genetic diversity of a crop species and its relatives in the entire collection with a minimal repetitiveness (Ortiz, 2002; Warburton *et al*, 2008). The core collections aim to maximize the genetic diversity (of a large collection) in a smaller number of accessions, while breeders usually only seek one or a few traits at a time when approaching a genetic resource collection (Mackay, 1995). A new approach called Focused

Identification of Germplasm Strategy (FIGS) was recently suggested (Mackay and Street, 2004) which can improve the efficiency of accessing the genetic resources. The central idea behind FIGS is to focus on the trait being sought and identify germplasm from collections sites where there would have been a selection pressure for the trait. We have discussed the successful use of FIGS approach to identify a subset of 1320 landraces from 16'089 accessions held in three different gene banks. Sixteen percent of these landraces were resistant when challenged with four different isolates and also showed high variation for the specific locus *Pm3*.

The genetic diversity held in gene banks also remains largely unexplored at molecular level, due to lack of fast and efficient tools to identify and study potentially useful new alleles. The development of molecular tools to specifically access the existing genetic diversity at particular loci facilitates the rapid analysis of allelic diversity in the gene pool of wheat. Allele mining seems to be a promising, although largely untested method to unlock the diversity in the genetic collections. To design precise and targeted molecular tools for diversity analysis based on allele mining, knowledge on the DNA sequence at a particular resistance locus is important, requiring the molecular cloning of genes. DNA sequence information is essential to devise rapid and inexpensive PCR strategies to isolate alleles of identified resistance genes from a wide range of cultivars, landraces and related species. This allows the molecular isolation of undisclosed allelic variants with potential agronomical relevance and a more efficient and targeted use of genetic resources for research and breeding.

There are reports about the use of allele mining strategy in several cereal species to isolate alleles of target genes. In barley, an evaluation of cultivated germplasm was carried out to detect the presence of thermostable alleles of β-amylase (*Bmy-Sd2H* and *Bmy-Sd3* alleles) that

improves the fermentability during brewing (Malysheva *et al*, 2004). The study carried out on 891 accessions led to the identification of 166 accessions with superior alleles, suggesting that the improvement of malting quality in barley could be achieved by introducing these alleles into breeding programs. There is an ongoing study in maize to identify alleles for *lcyE* gene and other genes that increase total carotenoids and that slow down the conversion of β-carotene to β-cryptoxanthin and zeaxanthin (Harjes et al, 2008). Barley germplasm is being screened to identify allelic variants of *Hv-eiF4E* which imparts virus resistance (Stein *et al*, 2007). Latha *et al* (2004) reported development of markers for allele mining of stress tolerance genes in rice germplasm. The new HMW-glutenin alleles encoded by the *Glu-R1* locus of *Secale cereale* (rye) have been analysed and characterized (De Bustos & Jouve, 2003) from different rye cultivars and their most closely related wild subspecies.

We are not aware of such allele mining projects for disease resistance in wheat. As the first wheat disease resistance genes have been cloned (Huang *et al*, 2003; Feuillet *et al*, 2003; Yahiaoui *et al*, 2004; Yahiaoui *et al*, 2006; Cloutier *et al*, 2007), the sequence information of these genes could allow the rapid analysis of the genetic diversity at these loci over a wide range of germplasm and also the subsequent identification of new alleles through allele mining.

The approach we described is one of the first large-scale attempts of a systematic resistance allele-mining from wheat landraces using molecular tools derived from the target gene sequence. We focused on the *Pm3* resistance locus because of the precise sequence information available for targeted allele cloning. For characterization of the "FIGS powdery mildew set" we used a combined strategy of screening for genetic diversity with molecular markers and classical pathogenicity tests. We characterised the lines for resistance against

powdery mildew as well as for the occurrence of already known *Pm3* alleles by using allele specific markers. *Pm3b* was found to be the most frequent allele, followed by *Pm3*c while the other alleles known from the literature were not found in the subset. *Pm3b* was identified in landraces originating from Afghanistan, Iran, Russia, Azerbaijan and Turkey. The first identification of the *Pm3b* allele was in a landrace from Uzbekistan (http://www.ars-grin.gov/npgs/index.html), which is consistent with its frequency and actual geographical distribution particularly in Afghanistan, a neighbouring country to Uzbekistan. This molecular analysis also led us to identification of candidate powdery mildew resistance lines which possess a *Pm3*-like gene but not a known allele of *Pm3*, hence, making them the best candidates for isolation of new *Pm3* alleles. We have identified 16 new *Pm3* alleles. Among these, the seven functionally active *Pm3* alleles, *Pm3l*, *Pm3m*, *Pm3n*, *Pm3o*, *Pm3p*, *Pm3q* and *Pm3r* now extend the previously known *Pm3* allelic series.

We have described the use of allele mining and efficient screening of gene banks for identification of allelic variants at the *Pm3* locus of wheat. The strategy we describe here is successful and can be implemented for other diversity and molecular breeding studies involving agronomically important traits. However, the limitation of this approach in wheat lies in the fact that very few genes of agronomical importance have yet been cloned. This is particularly true for genes involved in disease resistance.

2. Sequence diversity at the *Pm3* locus and identification of new functional *Pm3* variants

The sequence analysis of the newly isolated alleles revealed an identical gene structure and a high level of sequence identity over the entire length of alleles. It was this conservation of *Pm3* gene structure that allowed the isolation of 16 and 24 new *Pm3* alleles from hexaploid

landraces and tetraploid wheat species, respectively. This indicates that no large re-arrangements have affected the *Pm3* locus. The differences of the new alleles in comparison to *Pm3*CS or known *Pm3* alleles were mainly found in the LRR domain, with an exception of a few alleles which possess polymorphism also in the Interspacer (between NBS and LRR domain) and NBS domains. In other studies, sequence variability in *R* genes/alleles was mostly found in the LRR region which was shown to play a major role in pathogen recognition specificity (e.g. Ellis *et al*, 1999, Shen *et al*, 2001, Yahiaoui *et al*, 2006). In case of flax rust resistance locus *L*, the major sequence and structural variation among the *L* alleles was also found in LRR domain (Ellis et al, 1999). In contrast, the 100% conservation of the N-terminal region that encodes for a coiled coil domain among the new and already reported *Pm3* alleles, suggests a highly conserved function of this domain in *Pm3* resistance.

The alleles show polymorphisms in the sequences which probably are the result of re-arrangement of variation present in the ancestral alleles. Some alleles have defined polymorphic blocks in the LRR region which might have arisen from gene conversion or recombination events. Sequence exchange through gene conversion seems also to be a major mechanism for *Pm3* evolution in both tetraploid and hexaploid wheat. *R* genes evolve through a variety of molecular mechanisms: point mutations, unequal crossing over, gene conversion and recombination, illegitimate recombination and insertion/deletions all contribute to variability (Michelmore and Meyers, 1988; Kuang *et al*, 2004, Wicker *et al*, 2007). The new hexaploid *Pm3* alleles also show differences in their coding regions which originated due to InDels. InDELs were as well found in the functional flax *L* alleles (Ellis et al, 1999) and among the various members of *Mla* family (Wei et al, 2002). Deletions found in the new *Pm3* alleles reported here might have originated from illegitimate recombination,

which has been proposed to be new major evolutionary mechanism that is at the basis of variability of LRR domain of *R* genes (Wicker et al, 2007).

The seven new *Pm3* alleles (now *Pm3l* to *Pm3r*) isolated from hexaploid landraces and one *Pm3* allele *Pm3k* from tetraploid wheat showed significant reduction in the haustorium index as compared to the susceptible control *Pm3CS*, in the transient expression assay. Two of the hexaploid *Pm3* alleles *Pm3_42416* and *Pm3_42255* were also silenced through VIGS. In the landraces possessing these two alleles, the resistance is completely controlled by *Pm3* alleles which allowed a detailed further study on resistance activity and specificity. The intermediate haustorium indices of these alleles observed in transient assay was confirmed by the LPTB staining, which demonstrated that important resistance activity of the alleles occur later than 48hrs, the time point when staining and observation is made in the transient assay. We infer that at least two of these new alleles isolated from landraces are slow acting alleles while the known *Pm3* alleles are known to provide a rapid resistance response without formation of haustoria (Yahiaoui et al, 2004; this study). This opens up a discussion if slow resistance is a general phenomenon of *Pm3* genes in landraces and the reason why these genes were never selected for breeding. A late hypersensitive cell death associated resistance has also been reported for barley powdery mildew resistance genes *Mla3* and *Mla7* (Boyd *et al*, 1995; Shen *et al*, 2003) and might be a widespread phenomenon in allelic series of *R* genes. The other diverse *Pm3* sequences which were not found functional in the present study could be further investigated by using additional *Bgt* isolates taking into account the race-specificity of *Pm3* alleles.

3. Origin and evolution of *Pm3* locus in wild tetraploid wheat and hexaploid wheat

The comparative analysis of disease resistance genes which have evolved in parallel in wild and domesticated species allows the study of molecular evolutionary mechanisms creating functional diversity in resistance. In view of this, we screened a collection of wild and domesticated tetraploid wheat lines for *Pm3* genes and studied their genetic and geographical differentiation. The identification and the conservation of the *Pm3* locus at two different ploidy levels in the wheat gene pool allowed to correlate and to link the evolution of this gene in relation to the history of wheat evolution and domestication. Among the 3 main tetraploid gene pools (wild *T. dicoccoides*, domesticated *T. dicoccum* and *T. durum*) screened for their *Pm3* haplotype, most *Pm3* alleles were detected in wild *T. dicoccoides* accessions showing that the wild gene pool is enriched in *Pm3* sequences compared to the domesticated emmer.

Phylogenetic analysis of *Pm3* haplotypes from tetraploid wheat identified two main clades differing by one sequence block of ancient origin with a divergence time corresponding to the divergence of ancestral wheat diploid species (Huang *et al*, 2002; Dvorak *et al*, 2006). In hexaploid bread wheat *Pm3* alleles, no synonymous mutations were found outside of clearly delimited polymorphic sequence blocks (Yahiaoui *et al*, 2006) leading to the hypothesis of a very recent divergence 10,000 years ago. In tetraploid wheat, few synonymous mutations were found outside of the polymorphic blocks. This likely reflects a relatively more ancient divergence compared to bread wheat alleles and is compatible with the estimated time of the first hybridization event at the origin of tetraploid wheat, less than 0.5 MYA (Huang *et al*, 2002).

Functional *Pm3* resistance alleles are not very frequent in the tetraploid gene pool although it was possible to identify functional *Pm3* allele, now called *Pm3k*. The low frequency of *Pm3*-based resistance in wild emmer is similar to the situation in lettuce where the *Dm3* resistance gene against *Bremia lactucae* was rare in natural populations of the wild lettuce *Lactuca serriola* (Kuang *et al*, 2006). We assume that low frequency of *Pm3*-based resistance in wild emmer could be due to a recent and independent evolution of functional *Pm3* resistance genes in the tetraploid vs. hexaploid wheat species. Until now only the *Pm3CS* allele was found identical between bread wheat and tetraploid wheat. In bread wheat, the *Pm3* resistance alleles diverged from the *Pm3CS* template either by few specific point mutations or by gene conversion events introducing polymorphic sequence blocks which are different from the ones found in tetraploid wheat (Yahiaoui *et al*, 2006).

4. Outlook

We demonstrate that FIGS is an effective sampling strategy that could be applied to other disease resistance screening studies as well to more efficiently mine gene bank collections for specific traits. Allele mining offers a relatively easy and appropriate alternative to identify functional alleles as compared to traditional ways of identifying new sources of resistance. Traditionally, resistance genes in wild relatives of wheat have been introgressed by complex breeding schemes involving irradiation and chromosomal translocations (Baum *et al*, 1992). This resulted in the introgression of large chromosomal segments, often carrying negative breeding traits (linkage drag). The newly identified *Pm3* alleles here could be transferred by classical genetic crosses to powdery mildew susceptible cultivars or alternatively be transformed into susceptible varieties as single genes. In addition, they could be combined as

R gene cassettes to achieve disease control, as each of these *R* genes usually acts only against a subset of the existing pathogen races. Partial resistance conferred by the genes we identified could be of agricultural interest as it would potentially induce less pressure on the pathogen populations and would therefore be of more durable use.

Besides these more applied aspects in wheat breeding, the analysis of allelic diversity and accumulation of diverse allelic sequences will contribute to a better characterization of the mechanisms involved in resistance gene evolution. The identification of new functional *Pm3* alleles from diverse germplasm will also contribute to the molecular understanding of *R* gene function. The comparison of sequences from new alleles can clarify the molecular basis of *Pm3* specificity, e.g. by studying chimeric genes created by domain swap experiments with domains from the newly identified sequences.

VIII. References

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IX. Appendix

9.1 List of hexaploid landraces from which the *Pm3* alleles were isolated.

	Pm3 allele	Accession	Origin
1	*Pm3CS*	AUS 4856	Pakistan
2	*Pm3CS*	AUS 13655	Afghanistan
3	*Pm3CS*	AUS 13656	Afghanistan
4	*Pm3CS*	AUS 13704	Afghanistan
5	*Pm3CS*	AUS 14526	Afghanistan
6	*Pm3CS*	IG 41554	Pakistan
7	*Pm3CS*	IG 42398	Turkey
8	*Pm3CS*	IG 122584	Iran
9	*Pm3CS*	IG 42869	Turkey
10	*Pm3_9939*	AUS 9939	Afghanistan
11	*Pm3_10963*	AUS 10963	Afghanistan
12	*Pm3_13636*	AUS 13636	Afghanistan
13	*Pm3_13636*	IG 42326	Turkey
14	*Pm3_14442*	AUS 14442	Afghanistan
15	*Pm3_14475*	AUS 13645	Afghanistan
16	*Pm3_14475*	AUS 13706	Afghanistan
17	*Pm3_14475*	AUS 14444	Afghanistan
18	*Pm3_14475*	AUS 14475	Afghanistan
19	*Pm3_14475*	VIR 49003	Afghanistan
20	*Pm3_23728*	VIR 23728	Turkmenistan
21	*Pm3_31594*	VIR 31594	Azerbaijan
22	*Pm3_41606*	IG 41606	Pakistan
23	*Pm3_42255*	IG 42255	Turkey
24	*Pm3_42255*	IG 42453	Turkey
25	*Pm3_42255*	IG 42597	Turkey
26	*Pm3_42255*	IG 42895	Turkey
27	*Pm3_42255*	IG 43031	Turkey
28	*Pm3_42255*	VIR 35209	Turkey
29	*Pm3_42277*	IG 42263	Turkey
30	*Pm3_42277*	IG 42274	Turkey
31	*Pm3_42277*	IG 42277	Turkey
32	*Pm3_42277*	IG 43026	Turkey
33	*Pm3_42281*	IG 42281	Turkey
34	*Pm3_42416*	IG 42327	Turkey
35	*Pm3_42416*	IG 42378	Turkey
36	*Pm3_42416*	IG 42416	Turkey
37	*Pm3_42416*	IG 42431	Turkey
38	*Pm3-42525*	IG 42525	Turkey
39	*Pm3-42525*	VIR 20918	Turkey
40	*Pm3_42469*	IG 42432	Turkey
41	*Pm3_42469*	IG 42469	Turkey
42	*Pm3_42469*	VIR 14408	Turkey
43	*Pm3_42868*	IG 42374	Turkey
44	*Pm3_42868*	IG 42868	Turkey
45	*Pm3_42920*	IG 42920	Turkey

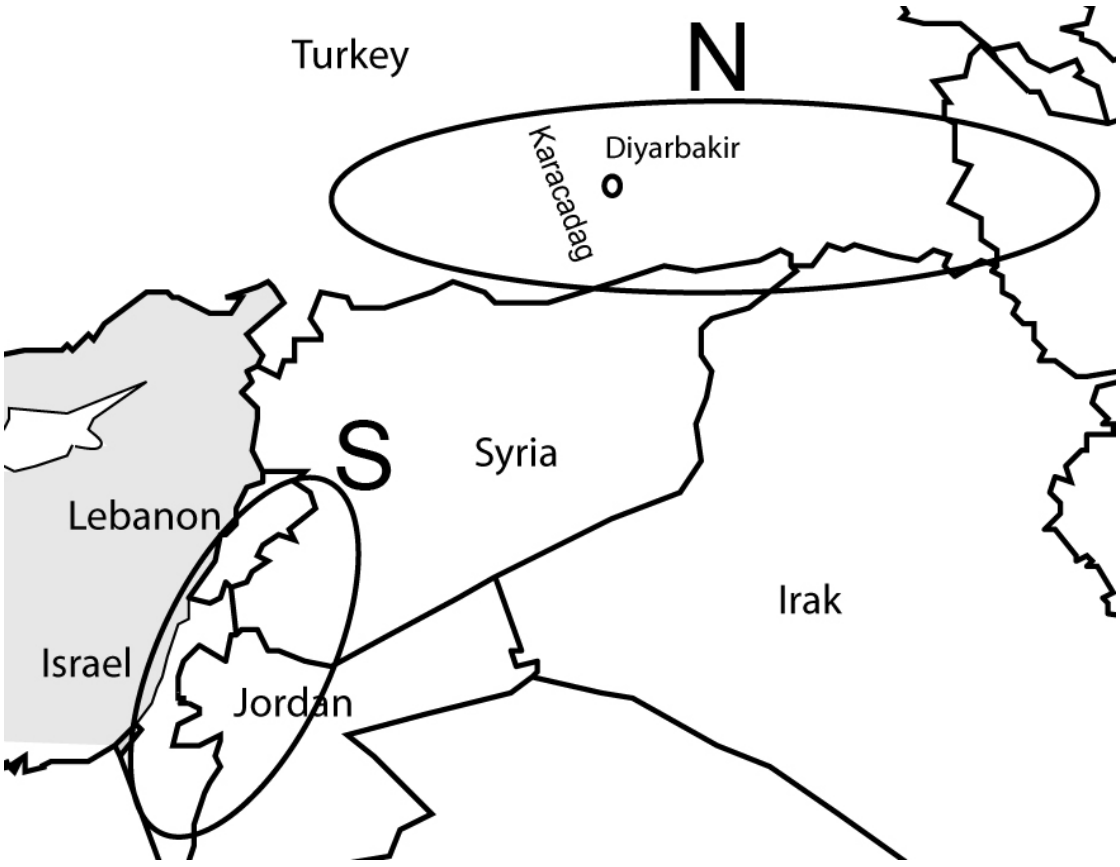
9.2 Phenotypic assay for powdery mildew resistance by infection with ten different isolates.

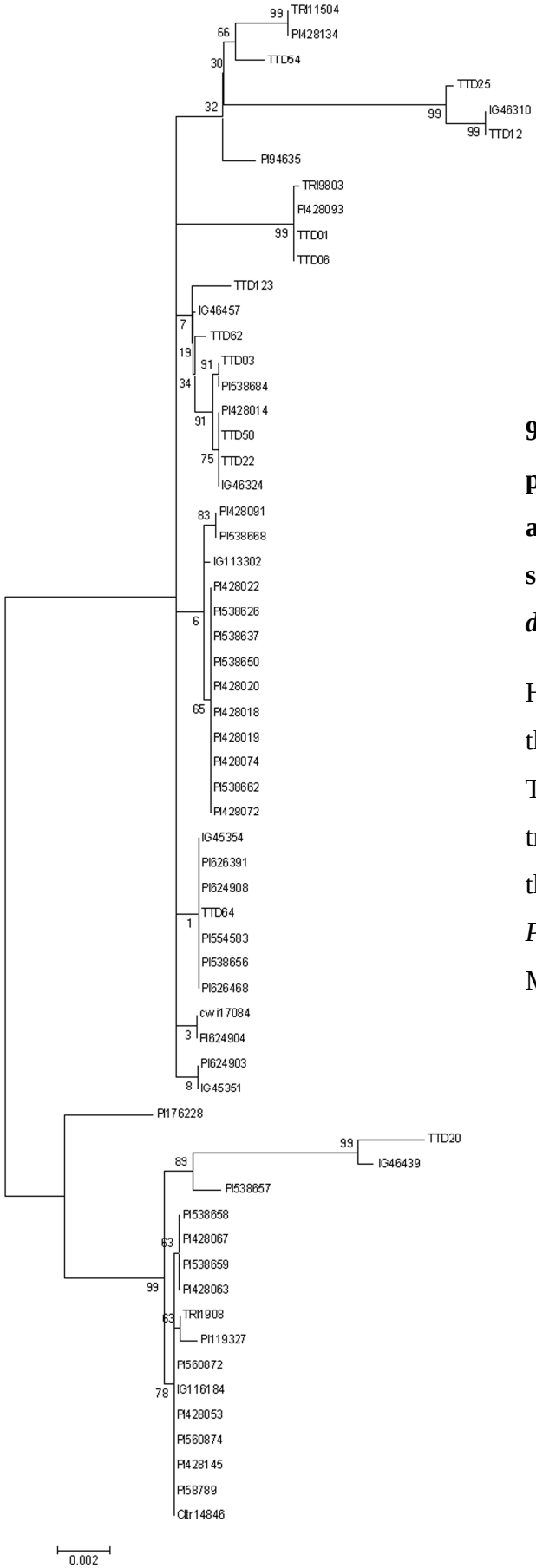
Lines with *Pm3* alleles	96224	96244	98275	Syros 2000.15	9530 M Dove	95.45 Brimstone	DB asosan	97011	97019	96229
Pm3a	R	R	R	S	R	R	S	R	R	R
Pm3b	R	R	R	S	R	R	S	S	R	R
Pm3c	R	R	R	S	RI	RI	S	S	R	R
Pm3d	R	R	R	S	R	R	R	R	S	S
Pm3e	IR	S	R	S	R	R	RI	I	R	I
Pm3f	R	R	R	S	R	I	S	**	R	R
Pm3g	S	R	S	S	S	S	R	S	IS	S
Pm3_42416	R	IS	R	S	R	R	IS	R	S	R
Pm3_42255	R	S	R	S	R	IS	I	R	S	R

** Data not available

9.3 Map of the Fertile Crescent region from which the wild emmer wheat accessions originate.

The Northern and Southern population range are indicated.





9.4 Neighbour-joining phylogenetic tree of *Pm3* alleles from tetraploid wheat species (*T.dicoccoides, T. dicoccum* and *T. durum*).

Haplotypes corresponding to the accessions are indicated in Table 9.5. The phylogenetic tree was inferred on the basis of the amino acid sequences of the *Pm3* alleles in the program Mega version 4.

9.5 Differential reactions to powdery mildew infection and characterization of the *Pm3* haplotypes in 61 accessions of wild and cultivated tetraploid wheat lines.

Different haplotype numbers (H1 to H24) correspond to different *Pm3* allelic sequences.

Accession	Species	Status	Origin	*Pm3* haplotype	Powdery mildew Isolate		
					96224	97019	DB ASOSAN
PI538656	*T. dicoccoides*	W	Turkey, Karacadag	H1	S	S	S
PI 554583	*T. dicoccoides*	W	Turkey, Urfa	H1	S	S	S
TTD64	*T. dicoccoides*	W	Turkey	H1	S	S	S
cwi17084	*T. dicoccum*	D	Irak	H1	R(HR)	R(HR)	R(HR)
IG-45351	*T. dicoccum*	D	Iran	H1	S	S	IR
IG-45354	*T. dicoccum*	D	Russia	H1	R	R	R
PI626391	*T. dicoccum*	D	Iran, Esfahan	H1	S	IS	IS
PI624903	*T. dicoccum*	D	Iran, Kordestan	H1	S	IS	IS
PI624904	*T. dicoccum*	D	Iran, Kordestan	H1	S	IS	IS
PI624908	*T. dicoccum*	D	Iran, Kordestan	H1	S	IS	IS
PI626468	*T. dicoccum*	D	Iran, Esfahan	H1	S	S	S
PI428018	*T. dicoccoides*	W	Turkey, Karacadag	H2	S	S	R
PI428019	*T. dicoccoides*	W	Turkey, Karacadag	H2	S	S	S
PI428020	*T. dicoccoides*	W	Turkey, Karacadag	H2	R	R	R
PI428022	*T. dicoccoides*	W	Turkey, Karacadag	H2	S	S	S
PI 428072	*T. dicoccoides*	W	Turkey, Karacadag	H2	S	S	S
PI428074	*T. dicoccoides*	W	Turkey,Karacadag	H2	S	S	S
PI538626	*T. dicoccoides*	W	Turkey,Karacadag	H2	IR(HR)	IR(HR)	S
PI538637	*T. dicoccoides*	W	Turkey,Karacadag	H2	S	S	S
PI538650	*T. dicoccoides*	W	Turkey,Karacadag	H2	S	S	S
PI538662	*T. dicoccoides*	W	Turkey,Karacadag	H2	S	S	IR
PI428091	*T. dicoccoides*	W	Turkey, Urfa	H3	S	S	R
PI538668	*T. dicoccoides*	W	Turkey, Urfa	H3	S	S	S
IG-113302	*T. dicoccoides*	W	Iran	H4	S	S	S
PI94635	*T. dicoccum*	D	Iran	H5	IS	IR	IR
TTD123	*T. dicoccoides*	W	Israel	H6	R(HR)	R	R
TTD62	*T. dicoccoides*	W	Israel	H7	R	R	R
TTD22	*T. dicoccoides*	W	Israel	H8	S	S	IR
TTD50	*T. dicoccoides*	W	Israel	H8	S	IR	IR
PI428014	*T. dicoccoides*	W	Israel	H8	R	R	R
IG-46324	*T. dicoccoides*	W	Jordan	H8	S	S	S
TTD54	*T. dicoccoides*	W	Israel	H9	R(HR)	R(HR)	R(HR)
TRI 11504	*T. dicoccoides*	W	Lebanon	H10	R	R	R
PI428134	*T. dicoccoides*	W	Lebanon	H10	S	S	S
TTD25	*T. dicoccoides*	W	Israel	H11	R	R	R
TTD12	*T. dicoccoides*	W	Israel	H12	S	S	S
IG-46310	*T. dicoccoides*	W	Palestine	H12	S	S	S
TTD01	*T. dicoccoides*	W	Israel	H13	ND	ND	ND
TTD06	*T. dicoccoides*	W	Israel	H13	R	R	R
PI428093	*T. dicoccoides*	W	Israel	H13	S	S	S

TTD20	*T. dicoccoides*	W	Israel	H14	R	R	R
TRI 9803	*T. dicoccoides*	W	Israel	H15	R	R	R
PI560872	*T. dicoccoides*	W	Turkey, Siirt	H16 (*Pm3CS*)	IS	IS	IR
PI560874	*T. dicoccoides*	W	Turkey, Siirt	H16 (*Pm3CS*)	R	R	R
PI428053	*T. dicoccoides*	W	Turkey, Karacadag	H16 (*Pm3CS*)	IS	R	R
PI428145	*T. dicoccoides*	W	Turkey, Mardin	H16 (*Pm3CS*)	R	R	I
IG116184	*T. dicoccoides*	W	Turkey, Gaziantep	H16 (*Pm3CS*)	R(HR)	R(HR)	R
PI58789	*T. dicoccum*	D	Ethiopia, Shewa	H16 (*Pm3CS*)	ND	ND	ND
CItr14846	*T. durum*	D	Ethiopia, Welo	H16 (*Pm3CS*)	S	S	S
TRI 1908	*T. durum*	D	Greece	H17	R	R	R
PI538658	*T. dicoccoides*	W	Turkey,Karacadag	H18	R(HR)	R	R
PI538659	*T. dicoccoides*	W	Turkey,Karacadag	H18	IR(HR)	R	R
PI428063	*T. dicoccoides*	W	Turkey, Karacadag	H18	IR(HR)	R	R(HR)
PI 428067	*T. dicoccoides*	W	Turkey,Karacadag	H18	R	R	R
PI538657	*T. dicoccoides*	W	Turkey,Karacadag	H19	S	S	S
IG-46457	*T. dicoccoides*	W	Syria	H20	S	S	S
TTD03	*T. dicoccoides*	W	Israel	H21	R	R	R
PI538684	*T. dicoccoides*	W	Israel	H21	R	R	R
IG-46439	*T. dicoccoides*	W	Syria	H22	R	R/IR	R
PI119327	*T. durum*	D	Turkey,Karacadag	H23	S	S	IS
PI176228	*T. durum*	D	Nepal	H24	S	S	IS

W, wild; D, domesticated R, full resistance; S full susceptibility; IR (HR) hypersensitive reaction and partial sporulation of the fungus are visible, IR, few sporulating colonies of the fungus are visible, IS intermediate susceptible(between 10 to 40% of leaf surface covered with sporulating colonies); ND not determined.

9.6 List of all tetraploid wheat accessions screened for their haplotype at the *Pm3* locus.

In the *Pm3* haplotype column, A is a positive *Pm3* marker, B is the presence of a band of different size possibly from the wheat B genome, 0 is no amplification.

PROVIDER	**Donor**	**Triticum turgidum subsp. dicoccoides**			***Pm3* haplotype**	***Pm3* gene**
		Donor Code				
		Accession	**Country**			
GRIN	USDA/ARS	PI 272582	Hungary, Pest	1	0	
GRIN	USDA/ARS	PI 346783	Hungary, Pest	2	0	
GRIN	USDA/ARS	PI 352322	Lebanon	3	B	
GRIN	USDA/ARS	PI 352326	Germany, Saxony-Anhalt	4	0	
GRIN	USDA/ARS	PI 352328	Germany, Saxony-Anhalt	5	B	
GRIN	USDA/ARS	PI 355456	Germany, Saxony-Anhalt	6	B	
GRIN	USDA/ARS	PI 355457	Germany, Saxony-Anhalt	7	0	
GRIN	USDA/ARS	PI 355458	Germany, N. Rhine-Westphal	8	B	
GRIN	USDA/ARS	PI 355459	Armenia	9	B	
GRIN	USDA/ARS	PI 428016	Iran, Bakhtaran	10	B	
GRIN	USDA/ARS	PI 428018	Turkey,Karacadag	11	AB	*Pm3*_H2
GRIN	USDA/ARS	PI 428019	Turkey,Karacadag	12	AB	*Pm3*_H2
GRIN	USDA/ARS	PI 428020	Turkey,Karacadag	13	A	*Pm3*_H2
GRIN	USDA/ARS	PI 428021	Turkey,Karacadag	14	AB	ND
GRIN	USDA/ARS	PI 428022	Turkey,Karacadag	15	AB	*Pm3*_H2
GRIN	USDA/ARS	PI 428091	Turkey, Urfa	16	AB	*Pm3*_H3
GRIN	USDA/ARS	PI 428092	Turkey, Karacadag	17	B	

GRIN	USDA/ARS	PI 428145	Turkey, Mardin	18	A	*Pm3CS*_H16
GRIN	USDA/ARS	PI 466991	Syria, Al Qunaytirah	19	0	
GRIN	USDA/ARS	PI 466992	Syria, Al Qunaytirah	20	0	
GRIN	USDA/ARS	PI 466994	Syria, Al Qunaytirah	21	B	
GRIN	USDA/ARS	PI 538665	Turkey, Karacadag	22	B	
GRIN	USDA/ARS	PI 538666	Turkey, Karacadag	23	B	
GRIN	USDA/ARS	PI 538667	Turkey, Urfa	24	B	
GRIN	USDA/ARS	PI 538668	Turkey, Urfa	25	AB	*Pm3*_H3
GRIN	USDA/ARS	PI 554580	Turkey, Urfa	26	AB	ND
GRIN	USDA/ARS	PI 554581	Turkey, Karacadag	27	B	
GRIN	USDA/ARS	PI 554583	Turkey, Urfa	28	AB	*Pm3*_H1
GRIN	USDA/ARS	PI 560697	Turkey, Siirt	29	B	
GRIN	USDA/ARS	PI 560872	Turkey, Siirt	30	AB	*Pm3CS*_H16
GRIN	USDA/ARS	PI 560874	Turkey, Siirt	31	AB	*Pm3CS*_H16
GRIN	USDA/ARS	PI 428134	Lebanon, El Beqaa	32	AB	*Pm3*_H10
GRIN	USDA/ARS	PI 538708	Lebanon, El Beqaa	33	B	
J.David INRA_21	Icarda	IG-113302	IRAN	34	A	*Pm3*_H4
J.David INRA_23	Icarda	IG-46391	JORDAN	35	0	
J.David INRA_25	Icarda	IG-46470	SYRIA	36	B	
J.David INRA_26	Icarda	IG-46253	TURKEY	37	0	
J.David INRA_27	Icarda	IG-116172	TURKEY	38	B	
J.David INRA_28	USDA	PI 467014	ISRAEL	39	B	
J.David INRA_29	USDA	PI 428133	LEBANON	40	B	
J.David INRA_30	Icarda	IG-46516	SYRIA	41	0	
J.David INRA_31	USDA	PI 487255	SYRIA	42	0	
J.David INRA_33	Icarda	IG-115811	JORDAN	43	B	
J.David INRA_34	Icarda	IG-46294	LEBANON	44	B	

J.David INRA_35	Icarda	IG-46310	PALESTINE	45	A	*Pm3*_H12
J.David INRA_36	Icarda	IG-46501	SYRIA	46	B	
J.David INRA_37	Icarda	IG-116179	TURKEY	47	B	
J.David INRA_38	Icarda	IG-116184	TURKEY	48	AB	*Pm3CS*_H16
J.David INRA_39	Icarda	IG-46191	TURKEY	49	AB	*Pm3*_H15
A. Graner	IPK,Gatersleben	TRI 1902	Europe	50	B	
A. Graner	IPK,Gatersleben	TRI 4467	Europe	51	B	
A. Graner	IPK,Gatersleben	TRI 9803	Israel	52	A	*Pm3*_H13
A. Graner	IPK,Gatersleben	TRI 11504	Lebanon	53	AB	*Pm3*_H10
A. Graner	IPK,Gatersleben	TRI 3421	Europe	54	0	
A. Graner	IPK,Gatersleben	TRI 3425	Europe	55	B	
M.Feldman		TTD01	Israel	56	AB	*Pm3*_H13
M.Feldman		TTD03	Israel	57	AB	*Pm3*_H21
M.Feldman		TTD04	Israel	58	0	
M.Feldman		TTD06	Israel	59	AB	*Pm3*_H13
M.Feldman		TTD07	Israel	60	0	
M.Feldman		TTD10	Israel	61	0	
M.Feldman		TTD12	Israel	62	A	*Pm3*_H12
M.Feldman		TTD14	Israel	63	B	
M.Feldman		TTD15	Israel	64	0	
M.Feldman		TTD20	Israel	65	A	*Pm3*_H14
M.Feldman		TTD21	Israel	66	AB	ND
M.Feldman		TTD22	Israel	67	A	*Pm3*_H8
M.Feldman		TTD23	Israel	68	B	
M.Feldman		TTD24	Israel	69	0	
M.Feldman		TTD25	Israel	70	A	*Pm3*_H11
M.Feldman		TTD28	Israel	71	B	

M.Feldman		TTD30	Israel	72	0	
M.Feldman		TTD37	Iran	73	B	
M.Feldman		TTD47	Israel	74	B	
M.Feldman		TTD48	Israel	75	B	
M.Feldman		TTD50	Israel	76	A	*Pm3*_H8
M.Feldman		TTD51	Israel	77	0	
M.Feldman		TTD54	Israel	78	AB	*Pm3*_H9
M.Feldman		TTD56	Israel	79	AB	No amplification
M.Feldman		TTD59	Israel	80	0	
M.Feldman		TTD60	Israel	81	B	
M.Feldman		TTD61	Israel	82	B	
M.Feldman		TTD62	Israel	83	AB	*Pm3*_H7
M.Feldman		TTD64	Turkey	84	AB	*Pm3*_H1
M.Feldman		TTD65	Israel	85	0	
M.Feldman		TTD69	Israel	86	B	
M.Feldman		TTD75	Israel	87	0	
M.Feldman		TTD96	Israel	88	0	
M.Feldman		TTD123	Israel	89	A	*Pm3*_H6
M.Feldman		TTD212	Israel	90	0	
GRIN	USDA/ARS	PI 428136	Lebanon, El Beqaa	91	B	
GRIN	USDA/ARS	PI 538710	Lebanon, El Beqaa	92	B	
GRIN	USDA/ARS	PI 428138	Lebanon, El Beqaa	93	B	
GRIN	USDA/ARS	PI 538711	Lebanon, El Beqaa	94	0	
GRIN	USDA/ARS	PI 428130	Lebanon, El Beqaa	95	B	
GRIN	USDA/ARS	PI 503316	Lebanon, El Beqaa	96	B	
GRIN	USDA/ARS	PI 538712	Lebanon, El Beqaa	97	B	
GRIN	USDA/ARS	PI 428066 G2130	Turkey. Karacadag	98	AB	ND

GRIN	USDA/ARS	PI 428067 G2132	Turkey. Karacadag	99	AB	*Pm3*_H18
GRIN	USDA/ARS	PI 428068 G2133	Turkey. Karacadag	100	AB	ND
GRIN	USDA/ARS	PI 428069 G2137	Turkey. Karacadag	101	AB	ND
GRIN	USDA/ARS	PI 428070 G2138	Turkey. Karacadag	102	AB	ND
GRIN	USDA/ARS	PI 428071 G2141	Turkey. Karacadag	103	AB	ND
GRIN	USDA/ARS	PI 428072 G2142	Turkey. Karacadag	104	AB	*Pm3*_H2
GRIN	USDA/ARS	PI 428073 G2143	Turkey. Karacadag	105	AB	ND
GRIN	USDA/ARS	PI 428074 G2144	Turkey. Karacadag	106	AB	*Pm3*_H2
GRIN	USDA/ARS	PI 428075 G2146	Turkey. Karacadag	107	AB	ND
GRIN	USDA/ARS	PI 428077 G2159	Turkey. Karacadag	108	B	
GRIN	USDA/ARS	PI 538637 G2069	Turkey. Karacadag	109	AB	*Pm3*_H2
GRIN	USDA/ARS	PI 538638 G2074	Turkey. Karacadag	110	AB	ND
GRIN	USDA/ARS	PI 538639 G2077	Turkey. Karacadag	111	AB	ND
GRIN	USDA/ARS	PI 538640 G2080	Turkey. Karacadag	112	A	ND
GRIN	USDA/ARS	PI 538642 G2082	Turkey. Karacadag	113	A	ND
GRIN	USDA/ARS	PI 538644 G2091	Turkey. Karacadag	114	B	
GRIN	USDA/ARS	PI 538650 G2105	Turkey. Karacadag	115	AB	*Pm3*_H2
GRIN	USDA/ARS	PI 538652 G2112	Turkey. Karacadag	116	AB	ND
GRIN	USDA/ARS	PI 538653 G2114	Turkey. Karacadag	117	AB	ND
GRIN	USDA/ARS	PI 538654 G2115	Turkey. Karacadag	118	AB	ND
GRIN	USDA/ARS	PI 538655 G2120	Turkey. Karacadag	119	AB	ND
GRIN	USDA/ARS	PI 538656 G2123	Turkey. Karacadag	120	AB	*Pm3*_H1
GRIN	USDA/ARS	PI 538657 G2124	Turkey. Karacadag	121	AB	*Pm3*_H19
GRIN	USDA/ARS	PI 538658 G2127	Turkey. Karacadag	122	AB	*Pm3*_H18
GRIN	USDA/ARS	PI 538662 G2139	Turkey. Karacadag	123	AB	*Pm3*_H2
GRIN	USDA/ARS	PI 538663 G2140	Turkey. Karacadag	124	AB	ND
F.Salamini		13520	Armenia	125	B	

F.Salamini		20455	Jordan	126	0	
F.Salamini		20457	USSR	127	B	
F.Salamini		43504	Israel	128	B	
F.Salamini		43506	Syria	129	0	
F.Salamini		43507	Syria	130	B	
F.Salamini		43508	Israel	131	B	
F.Salamini	Ozkan et al, 2002.	A TRI 3488	Europe	132	B	
F.Salamini	Ozkan et al, 2002.	17901	Israel	133	0	
F.Salamini	Ozkan et al, 2002.	PGR00061740101	Israel	134	AB	no amplification
F.Salamini	Ozkan et al, 2002.	PI 233288	Israel	135	B	
F.Salamini	Ozkan et al, 2002.	PI 352322	Lebanon	136	B	
F.Salamini	Ozkan et al, 2002.	PI 352324	Lebanon	137	0	
F.Salamini	Ozkan et al, 2002.	PI 414719	Israel	138	0	
F.Salamini	Ozkan et al, 2002.	PI 414720	Israel	139	0	
F.Salamini	Ozkan et al, 2002.	PI 414721	Israel	140	A	ND
F.Salamini	Ozkan et al, 2002.	PI 414722	Israel	141	B	
F.Salamini	Ozkan et al, 2002.	PI 428013	Israel	142	B	
F.Salamini	Ozkan et al, 2002.	PI 428014	Israel	143	A	*Pm3*_H8
F.Salamini	Ozkan et al, 2002.	PI 428015	Israel	144	B	
F.Salamini	Ozkan et al, 2002.	PI 428017	Turkey	145	B	
F.Salamini	Ozkan et al, 2002.	PI 428053	Turkey	146	AB	*Pm3CS*_H16
F.Salamini	Ozkan et al, 2002.	PI 428054	Turkey	147	B	
F.Salamini	Ozkan et al, 2002.	PI 428063	Turkey	148	AB	*Pm3*_H18
F.Salamini	Ozkan et al, 2002.	PI 428086	Turkey	149	B	
F.Salamini	Ozkan et al, 2002.	PI 428092	Turkey	150	B	
F.Salamini	Ozkan et al, 2002.	PI 428093	Israel	151	A	*Pm3*_H13
F.Salamini	Ozkan et al, 2002.	PI 428097	Israel	152	A	ND

F.Salamini	Ozkan et al, 2002.	PI 428099	Israel	153	AB	no amplification
F.Salamini	Ozkan et al, 2002.	PI 428100	Israel	154	0	
F.Salamini	Ozkan et al, 2002.	PI 428105	Israel	155	0	
F.Salamini	Ozkan et al, 2002.	PI 428126	Lebanon	156	B	
F.Salamini	Ozkan et al, 2002.	PI 428127	Lebanon	157	B	
F.Salamini	Ozkan et al, 2002.	PI 428132	Lebanon	158	A	no amplification
F.Salamini	Ozkan et al, 2002.	PI 428135	Lebanon	159	B	
F.Salamini	Ozkan et al, 2002.	PI 428143	Lebanon	160	0	
F.Salamini	Ozkan et al, 2002.	PI 466926	Israel	161	B	
F.Salamini	Ozkan et al, 2002.	PI 466949	Israel	162	0	
F.Salamini	Ozkan et al, 2002.	PI 466981	Israel	163	0	
F.Salamini	Ozkan et al, 2002.	PI 466991	Israel	164	0	
F.Salamini	Ozkan et al, 2002.	PI 466995	Israel	165	AB	no amplification
F.Salamini	Ozkan et al, 2002.	PI 467004	Israel	166	A	ND
F.Salamini	Ozkan et al, 2002.	PI 470988	Israel	167	B	
F.Salamini	Ozkan et al, 2002.	PI 471016	Israel	168	0	
F.Salamini	Ozkan et al, 2002.	PI 417035	Israel	169	AB	no amplification
F.Salamini	Ozkan et al, 2002.	PI 479780	Israel	170	A	no amplification
F.Salamini	Ozkan et al, 2002.	PI 487252	Syria	171	B	
F.Salamini	Ozkan et al, 2002.	PI 487253	Syria	172	AB	no amplification
F.Salamini	Ozkan et al, 2002.	PI 538626	Turkey	173	AB	*Pm3*_H2
F.Salamini	Ozkan et al, 2002.	PI 538633	Turkey	174	AB	
F.Salamini	Ozkan et al, 2002.	PI 538651	Turkey	175	AB	poor amplification
F.Salamini	Ozkan et al, 2002.	PI 538656	Turkey	176	AB	
F.Salamini	Ozkan et al, 2002.	PI 538657	Turkey	177	AB	
F.Salamini	Ozkan et al, 2002.	PI 538659	Turkey	178	AB	
F.Salamini	Ozkan et al, 2002.	PI 538680	Israel	179	A	

F.Salamini	Ozkan et al, 2002.	PI 538684	Israel	180	AB	*Pm3*_H21
F.Salamini	Ozkan et al, 2002.	PI 538685	Israel	181	0	
F.Salamini	Ozkan et al, 2002.	PI 538690	Israel	182	0	
F.Salamini	Ozkan et al, 2002.	PI 638699	Israel	183	0	
F.Salamini	Ozkan et al, 2002.	PI 538700	Lebanon	184	B	
F.Salamini	Ozkan et al, 2002.	PI 538719	Israel	185	0	
F.Salamini	Ozkan et al, 2002.	PI 554580	Turkey	186	AB	ND
F.Salamini	Ozkan et al, 2002.	PI 554581	Turkey	187	AB	ND
F.Salamini	Ozkan et al, 2002.	PI 554582	Turkey	188	AB	ND
F.Salamini	Ozkan et al, 2002.	PI 554583	Turkey	189	AB	*Pm3*_H1
F.Salamini	Ozkan et al, 2002.	PI 554584	Turkey	190	AB	ND
F.Salamini	Ozkan et al, 2002.	PI 503310	Turkey	191	AB	ND
F.Salamini	Ozkan et al, 2002.	PI 503312	Israel	192	0	
F.Salamini	Ozkan et al, 2002.	PI 503314	Israel	193	B	
F.Salamini	Ozkan et al, 2002.	PI 503315	Israel	194	B	
F.Salamini	Ozkan et al, 2002.	PI 503316	Lebanon	195	B	
F.Salamini	Ozkan et al, 2002.	IG 110737	Syria	196	AB	ND
F.Salamini	Ozkan et al, 2002.	IG 110815	Lebanon	197	AB	ND
F.Salamini	Ozkan et al, 2002.	IG 46526	Syria	198	B	
F.Salamini	Ozkan et al, 2002.	IG 46504	Syria	199	0	
F.Salamini	Ozkan et al, 2002.	IG 46473	Syria	200	B	
F.Salamini	Ozkan et al, 2002.	IG 46466	Syria	201	B	
F.Salamini	Ozkan et al, 2002.	IG 45492	Syria	202	B	
F.Salamini	Ozkan et al, 2002.	IG 45490	Syria	203	B	
F.Salamini	Ozkan et al, 2002.	IG 45493	Syria	204	0	
F.Salamini	Ozkan et al, 2002.	IG 45494	Syria	205	0	
F.Salamini	Ozkan et al, 2002.	IG 46320	Jordan	206	0	

F.Salamini	Ozkan et al, 2002.	IG 45502	Syria	207	B	
F.Salamini	Ozkan et al, 2002.	IG 46397	Syria	208	AB	ND
F.Salamini	Ozkan et al, 2002.	IG 45726	Jordan	209	0	
F.Salamini	Ozkan et al, 2002.	IG 46323	Jordan	210	A	poor amplification
F.Salamini	Ozkan et al, 2002.	IG 45676	Jordan	211	B	
F.Salamini	Ozkan et al, 2002.	IG 46324	Jordan	212	A	*Pm3*_H8
F.Salamini	Ozkan et al, 2002.	IG 46352	Jordan	213	B	
F.Salamini	Ozkan et al, 2002.	IG 45964	Jordan	214	AB	no amplification
F.Salamini	Ozkan et al, 2002.	IG46386	Jordan	215	AB	mixture
F.Salamini	Ozkan et al, 2002.	IG 46439	Syria	216	AB	*Pm3*_H22
F.Salamini	Ozkan et al, 2002.	IG 46457	Syria	217	AB	*Pm3*_H20
F.Salamini	Ozkan et al, 2002.	IG 46420	Syria	218	AB	ND
F.Salamini	Ozkan et al, 2002.	IG 46421	Syria	219	0	
		Triticum turgidum subsp. dicoccum				
		Accession	**Country**			
GRIN	USDA/ARS	PI 58789	Ethiopia, Shewa	1	AB	*Pm3CS*_H16
GRIN	USDA/ARS	Citr 14437	Ethiopia, Shewa	2	B	
GRIN	USDA/ARS	PI 197481	Ethiopia, Harer	3	B	
GRIN	USDA/ARS	PI 197482	Ethiopia, Harer	4	B	
GRIN	USDA/ARS	PI 197483	Ethiopia, Harer	5	B	
GRIN	USDA/ARS	PI 197484	Ethiopia, Harer	6	B	
GRIN	USDA/ARS	PI 273978	Ethiopia, Harer	7	B	
GRIN	USDA/ARS	PI 273979	Ethiopia, Harer	8	B	

GRIN	USDA/ARS	PI 273980	Ethiopia, Shewa	9	B	
GRIN	USDA/ARS	PI 331265	Ethiopia, Sidamo	10	B	
GRIN	USDA/ARS	PI 624903	Iran, Kordestan	11	AB	*Pm3*_H1
GRIN	USDA/ARS	PI 624904	Iran, Kordestan	12	AB	*Pm3*_H1
GRIN	USDA/ARS	PI 624908	Iran, Kordestan	13	AB	*Pm3*_H1
GRIN	USDA/ARS	PI 626391	Iran, Esfahan	14	AB	*Pm3*_H1
GRIN	USDA/ARS	PI 626468	Iran, Esfahan	15	AB	*Pm3*_H1
GRIN	USDA/ARS	PI 317495	Afghanistan, Bamian	16	B	
GRIN	USDA/ARS	PI 321737	Afghanistan, Kabul	17	B	
GRIN	USDA/ARS	PI 337643	Afghanistan	18	B	
GRIN	USDA/ARS	PI 347132	Afghanistan, Herat	19	B	
J. David INRA_1	CIMMYT	cwi17263	ROMANIA	20	B	
J. David INRA_2	USDA	PI 355489	France	21	0	
J. David INRA_3	USDA	PI 319869	TURKEY	22	B	
J. David INRA_4	CIMMYT	cwi17084	IRAK	23	AB	*Pm3*_H1
J. David INRA_5	Icarda	IG-45383	BULGARIA	24	B	
J. David INRA_6	Icarda	IG-45351	IRAN	25	AB	*Pm3*_H1
J. David INRA_7	Icarda	IG-45239	ITALY	26	0	
J. David INRA_8	Icarda	IG-45354	RUSSIA	27	AB	*Pm3*_H1
J. David INRA_9	Icarda	IG-45280	SLOVAKIA	28	0	
J. David INRA_10	Icarda	IG-45309	SLOVAKIA	29	B	
J. David INRA_11	USDA	PI 352365	GERMANY	30	B	
J. David INRA_12	USDA	PI 355484	SPAIN	31	0	
J. David INRA_13	USDA	PI 94635	IRAN	32	AB	*Pm3*_H5
J. David INRA_14	USDA	PI 415152	ISRAEL	33	B	
J. David INRA_15	USDA	PI 94648	ITALY	34	B	
J. David INRA_16	Icarda	IG_45318	AFGHANISTAN	35	B	

J. David INRA_17	USDA	PI 355504	ITALY	36	B	
J. David INRA_18	CIMMYT	cwi18198	TURKEY	37	B	
J. David INRA_19	CIMMYT	cwi16932	BULGARIA	38	0	
J. David INRA_20	USDA/ARS	PI 286061	POLAND	39	B	
A. Graner	IPK, Gatersleben	TRI 586	Europe	40	B	
A. Graner	IPK, Gatersleben	TRI 1514	Europe	41	B	
A. Graner	IPK, Gatersleben	TRI 1564	Europe	42	B	
A. Graner	IPK, Gatersleben	TRI 1566	Europe	43	B	
A. Graner	IPK, Gatersleben	TRI 2022	Germany	44	B	
A. Graner	IPK, Gatersleben	TRI 2228	Europe	45	B	
A. Graner	IPK, Gatersleben	TRI 2246	Switzerland	46	B	
A. Graner	IPK, Gatersleben	TRI 2261	Europe	47	B	
A. Graner	IPK, Gatersleben	TRI 2883	Germany	48	B	
A. Graner	IPK, Gatersleben	TRI 2967	Europe	49	B	
A. Graner	IPK, Gatersleben	TRI 3418	Europe	50	?B	
A. Graner	IPK, Gatersleben	TRI 3423	Germany	51	B	
A. Graner	IPK, Gatersleben	TRI 4061	Europe	52	B	
A. Graner	IPK, Gatersleben	TRI 4313	Morocco	53	?B	
F.Salamini	Ozkan et al, 2002.	LEONESSA 2	Italy	54	B	
F.Salamini	Ozkan et al, 2002.	LEONESSA 5	Italy	55	0	
F.Salamini	Ozkan et al, 2002.	33	Italy	56	0	
F.Salamini	Ozkan et al, 2002.	AGNONE INVERNALE	Italy	57	0	
F.Salamini	Ozkan et al, 2002.	AGNONE PROMAVERILE	Italy	58	0	
F.Salamini	Ozkan et al, 2002.	POTENZA	Italy	59	B	
F.Salamini	Ozkan et al, 2002.	RIOFREDDO	Italy	60	0	

F.Salamini	Ozkan et al, 2002.	BGRC 11025	India	61	B	
F.Salamini	Ozkan et al, 2002.	BGRC 11053	ND	62	B	
F.Salamini	Ozkan et al, 2002.	BGRC 43443	ND	63	B	
F.Salamini	Ozkan et al, 2002.	PI 306526	Romania	64	B	
F.Salamini	Ozkan et al, 2002.	PI 355454	Asia Minor	65	B	
F.Salamini	Ozkan et al, 2002.	ID 1120	Lebanon	66	B	
F.Salamini	Ozkan et al, 2002.	Lonigo 19	Italy	67	B	
		Triticum turgidum subsp. durum				
J. Dubcovsky	UC Davis	Langdon	USA	1	0	
A. Graner	IPK,Gatersleben	TRI 601	Greece	2	0	
A. Graner	IPK,Gatersleben	TRI 673	Greece	3	B	
A. Graner	IPK,Gatersleben	TRI 699	Turkey	4	B	
A. Graner	IPK,Gatersleben	TRI 756	Turkey	5	0	
A. Graner	IPK,Gatersleben	TRI 1815	Greece	6	0	
A. Graner	IPK,Gatersleben	TRI 1818	Greece	7	B	
A. Graner	IPK,Gatersleben	TRI 1823	Greece	8	0	
A. Graner	IPK,Gatersleben	TRI 1829	Greece	9	0	
A. Graner	IPK,Gatersleben	TRI 1845	Greece	10	0	
A. Graner	IPK,Gatersleben	TRI 1858	Greece	11	0	
A. Graner	IPK,Gatersleben	TRI 1859	Greece	12	0	
A. Graner	IPK,Gatersleben	TRI 1877	Greece	13	B	
A. Graner	IPK,Gatersleben	TRI 1881	Greece	14	0	
A. Graner	IPK,Gatersleben	TRI 1893	Greece	15	B	
A. Graner	IPK,Gatersleben	TRI 1894	Greece	16	B	
A. Graner	IPK,Gatersleben	TRI 1908	Greece	17	A	*Pm3CS*_like_H16

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A. Graner	IPK,Gatersleben	TRI 1914	Greece	18	B	
A. Graner	IPK,Gatersleben	TRI 2088	Greece	19	0	
A. Graner	IPK,Gatersleben	TRI 2096	Albania	20	B	
A. Graner	IPK,Gatersleben	TRI 2097	Albania	21	B	
GRIN	USDA/ARS	PI 24605 CItr 4370	Turkey, Izmir	22	B	
GRIN	USDA/ARS	PI 177907 Ala	Turkey, Kocaeli	23	B	
GRIN	USDA/ARS	PI 117423 Red Harran	Turkey, Gaziantep	24	0	
GRIN	USDA/ARS	PI 119323 1523	Turkey, Urfa	25	B	
GRIN	USDA/ARS	PI 119326 1573	Turkey, Urfa	26	0	
GRIN	USDA/ARS	PI 119327 1613	Turkey, Karacadag	27	AB	*Pm3CS*_like
GRIN	USDA/ARS	CItr 14152 337	Ethiopia	28	A	ND
GRIN	USDA/ARS	CItr 14434 377b	Ethiopia	29	B	
GRIN	USDA/ARS	CItr 14436 378b	Ethiopia	30	0	
GRIN	USDA/ARS	CItr 14439 421b	Ethiopia	31	B	
GRIN	USDA/ARS	CItr 14844 ELS 6404-137-2	Ethiopia	32	AB	no amplification
GRIN	USDA/ARS	CItr 14846 ELS 6404-138-1	Ethiopia	33	AB	*Pm3CS*_H16
GRIN	USDA/ARS	CItr 14899 ELS 6404-158-3	Ethiopia	34	AB	no amplification
GRIN	USDA/ARS	PI 176228 9570a	Nepal	35	AB	*PM3*_H17
GRIN	USDA/ARS	PI 245661 3291	Afghanistan	36	B	
F. Salamini	Ozkan et al, 2002.	Aristan	France	37	0	
F. Salamini	Ozkan et al, 2002.	Aziziah	Palestine	38	B	
F. Salamini	Ozkan et al, 2002.	Baio	Italy	39	B	

F. Salamini	Ozkan et al, 2002.	Cappelli	Italy	40	B	
F. Salamini	Ozkan et al, 2002.	Duilio	Italy	41	B	
F. Salamini	Ozkan et al, 2002.	Coll.Jordan	Jordan	42	0	
F. Salamini	Ozkan et al, 2002.	Ofanto	Italy	43	0	
F. Salamini	Ozkan et al, 2002.	Razzak	Tunisia	44	B	
F. Salamini	Ozkan et al, 2002.	Roqueno	Spain	45	B	
F. Salamini	Ozkan et al, 2002.	Santa	Greece	46	B	
F. Salamini	Ozkan et al, 2002.	Simeto	Italy	47	B	
F. Salamini	Ozkan et al, 2002.	Siyah Kilakli	Turkey	48	A	poor amplification
F. Salamini	Ozkan et al, 2002.	Taganrog	Ukraine	49	B	
F. Salamini	Ozkan et al, 2002.	Triminia	Italy	50	B	
F. Salamini	Ozkan et al, 2002.	Tripolino	Italy	51	B	
F. Salamini	Ozkan et al, 2002.	Vatan	Tajikistan	52	B	
F. Salamini	Ozkan et al, 2002.	Villemur	France	53	B	
F. Salamini	Ozkan et al, 2002.	Waha	Mexico	54	B	
F. Salamini	Ozkan et al, 2002.	Bufala	Italy	55	B	
F. Salamini	Ozkan et al, 2002.	Gitit	?	56	0	
F. Salamini	Ozkan et al, 2002.	Pavone	Italy	57	B	
F. Salamini	Ozkan et al, 2002.	Russello	Italy	58	B	
F. Salamini	Ozkan et al, 2002.	Timilia	Italy	59	0	
F. Salamini	Ozkan et al, 2002.	CGN 06451	Italy	60	B	
F. Salamini	Ozkan et al, 2002.	MONTELEONE	Italy	61	0	
		Triticum turgidum subsp. carthlicum				
		2801	Sweden	1	B	

		2814	Turkey	2	B	
		2821	Turkey	3	B	
		2824	Turkey	4	0	
		2827	Turkey	5	B	
		2830	Turkey	6	B	
		2831	Turkey	7	B	
		2833	Turkey	8	B	
		2835	Turkey	9	B	
		2837	USA	10	B	
		2838	USA	11	0	
		2839	ex USSR	12	B	
		2830	Russia	13	B	
		2841	ex USSR	14	B	
		2844	USA	15	B	
		2847	Georgia	16	B	
		2849	Georgia	17	B	
		2854	Georgia	18	B	
		2856	Georgia	19	B	
		2857	Iran	20	B	
		2859	Iran	21	B	
		2861	Russia	22	B	
		2865	Russia	23	B	
		2868	Spain	24	B	
		2869	USA	25	B	
		2871	Poland	26	B	
		2872	ex USSR	27	B	

		2874	ex USSR	28	B	
		2879	United Kingdom	29	0	
		2884	USA	30	B	
		Triticum turgidum subsp. turanicum				
GRIN	USDA/ARS	PI125351	Afghanistan, Laghman	1	0	
GRIN	USDA/ARS	PI 127106	Afghanistan, Faryab	2	0	
GRIN	USDA/ARS	PI 283795	Afghanistan	3	B	
GRIN	USDA/ARS	PI 317495	Afghanistan, Bamian	4	B	
GRIN	USDA/ARS	PI 321737	Afghanistan, Kabul	5	B	
GRIN	USDA/ARS	PI 337643	Afghanistan	6	B	
GRIN	USDA/ARS	PI 347132	Afghanistan, Herat	7	B	

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Navreet Kaur

Curriculum- vitae

Last Name: **KAUR**
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Education and Qualifications:

1995	Matric (10^{th}) from Indian Certificate of Secondary Education, India.
1995-1997	Senior Secondary (10+2) from Punjab School Education Board, India.
1997-2000	B.Sc. (Medical) with Chemistry, Botany, Zoology, English & Punjabi from Guru Nanak Dev University, Amritsar, India.
2000-2002	M.Sc. (Genetics) with 8.32/10.00, from Punjab Agricultural University, Ludhiana, India. Title of M.Sc Thesis: "Genetic architecture for seed yield and quality in durum wheat (Triticum *durum* Desf.)"
2003-2004	Fellowship, P.A.U. Ludhiana, India.
2004-2008	PhD student at Institute of Plant Biology, University of Zurich, Zurich.

Publications:

- **Navreet Kaur**, Kenneth Street, Michael Mackay, Nabila Yahiaoui and Beat Keller. (2008) Molecular approaches for characterization and use of natural disease in wheat. European Journal of Plant Pathology, 121, 387-397.
- Paramjit Singh, **Navreet Kaur** and G S Mahal. 2005. Gene Effects for Quality Parameters in *Triticum durum.* Indian J. Genet. **65** (2): 115-116.
- **Navreet Kaur** and Paramjit singh. 2004. Gene effects for grain yield and related attributes in *Triticum durum*. Indian J.Genet. **64** (2): 137-138.

Conference Participations:

- Oral presentation at International Wheat Genetics Symposium, 25-29August 2008, Brisbane, Australia.
- Poster presentation at the North American Wheat Workers Workshop (NAWWW) held in Saskatoon, Saskatchewan, Canada. March 12 -14, 2007.
- Poster presentation at 'XIII International Congress on Molecular Plant-Microbe Interactions' held in Sorrento, Italy. July 21 - 27, 2007.
- Poster presentation in symposium "Feeding the Needs of Tomorrow- Plant Sciences from Basics to Application" organized by Zurich–Basel Plant Science centre on 31st March 2006.
- Delivered two lectures in training programme entitled "Biotechnology in crop improvement" held from March 9- 29, 2004 at Punjab Agricultural University, Ludhiana, India.

Awards and Distinctions:

- First award for poster presentation at 'North American Wheat Workers Workshop' held at Saskatoon, Canada, in March 2007.
- Qualified 'Agricultural Scientist recruitment board (Indian) National eligibility test (NET) in Plant breeding'.
- Awarded University Merit Fellowship and University certificate of merit (M.Sc).
- Award of Honour in Academics for M.Sc Genetics.